Functional Characterization of the Interaction of PHD2 with FKBP38

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1 Abbreviations

AKT	Synonym for protein kinase B
ANG	Angiopoietin
ANT	Adenine nucleotide translocase
Apaf-1	Apoptotic protease activating factor-1
APP	Amyloid precursor protein
AQP-1	Aquaporin-1
ARD1	Arrest-defective-1 protein
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATF4	Activating transcription factor-4
Bcl-2	B-cell CLL/Lymphoma 2
bHLH	Basic helix-loop-helix
CAIX	Carbonic anhydrase 9
CaM	Calmodulin
CBP	CREB-binding protein
Cdr2	Cerebellar degeneration-related protein 2
CFTR	Cystic fibrosis transmembrane conductance regulator
CH-domain	Cysteine/histidine rich domain
CHX	Cycloheximide
CRM1	Exportin 1
CsA	Cyclosporin A
CTAD	C-terminal transactivation domain
Cyp	Cyclophilin
Cyt *c*	Cytochrome *c*
DFX	Desferroxamine
DMOG	Dimethyloxalylglycine
DSS	Dextran-sodium sulphate
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein
ED	Embryonic day
EGL-9	Egg-laying abnormal-9
eIF4E	Eukaryotic initiation factor 4E
EPAS-1	Endothelial PAS protein 1
EPO	Erythropoietin
ETC	Electron transfer chain
FAD	Familial Alzheimer disease
FH	Fumarate hydratase
FIH	Factor inhibiting HIF
FKBP	FK506-binding protein
FRET	Fluorescence resonance energy transfer
Gli	Glioma associated oncogene homolog
GLUT1	Glucose transporter 1
HBS	HIF-binding site
HERG	Potassium voltage-gated channel subfamily H member 2
HIF	Hypoxia-inducible factor
HNSCC	Head and neck squamous cell carcinoma
HRE	Hypoxia-response element
HSP70/90	Heat shock protein 70/90
IκB	Inhibitor of NFκB
IKK	IκB kinase
IOP1	Iron-only hydrogenase-like protein
ING4	Inhibitor of growth family member 4
IPAS	Inhibitory PAS protein

IRES	Internal ribosome entry site
K_m	Michaelis-Menten constant
LDHA	Lactate dehydrogenase A
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
Mdm2	Mouse double minute 2
MEF	Mouse embryonic fibroblast
MLCK	Myosine light chain kinase
MMP9	Matrix metalloproteinase 9
MORG1	MAPK organizer 1
MPTP	Mitochondrial permeability transition pore
MYND	Myeloid, nervy and DEAF-1
MW	Molecular weight
NDRG1	N-myc downstream-regulated gene 1
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor-kappa B
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NQO1	NAD(P)H quinone oxidoreductase 1
NTAD	N-terminal transactivation domain
ODDD	Oxygen-dependent degradation domain
OS9	Amplified in osteosarcoma 9
PAS	PER-ARNT-SIM
PCNA	Proliferating cell nuclear antigen
PDH	Pyruvate dehydrogenase
PDK1/4	Pyruvate-dehydrogenase kinase 1/4
PER	Period circadian protein
PGK	Phosphoglycerate kinase
PHD	Prolyl-4-hydroxylase
PKA	Protein kinase A
PI3K	Phosphatidylinositol-3-kinase
PML	Promyelocytic leukaemia protein
pO_2	Oxygen partial pressure
PPIase	Peptidyl prolyl *cis/trans* isomerase
Ptch1	Patched 1
PTB	Polypyrimidine tract binding protein
PTEN	Phosphatase and tensin homolog
pVHL	Von Hippel-Lindau tumor suppressor protein
RACK1	Receptor of activated protein C kinase 1
REDD1	Regulated in development and DNA damage response 1 protein
Rheb	Ras homolog enriched in brain
RNAi	RNA interference
ROS	Reactive oxygen species
RP1	RNA polymerase 1
S6K	Ribosomal protein S6 kinase
SDH	Succinate dehydrogenase
SENP	Sentrin/SUMO-specific protease
SHH	Sonic hedgehog protein
SIAH	Seven in absentia drosophila homolog
SIM	Single-minded protein
Smo	Smoothenend
SSAT1	Spermidine/spermine-N-acetyltransferase-1

SUMO	Small ubiquitin-related modifier
SDC1	Syndecan 1
TAD	Transactivation domain
TCA	Tricarboxylic acid cycle (Krebs cycle)
TGFβ	Transforming growth factor β
TNF	Tumor necrosis factor
5'-TOP	5'-terminal oligopyrimidine tract
TPEN	N,N,N,N'-tetrakis (2-pyridylmethyl) ethylendiamine
TPR	Tetratricopeptide repeat
TriC	TCP1 ring complex polypeptide
TSC1/2	Tuberous sclerosis complex 1/2 tumor suppressor protein
UTR	Untranslated region
VDAC	Voltage-dependent anion transporter (VDAC)
VEGF	Vascular endothelial growth factor

2 Summary

Multicellular complex organisms utilize oxygen as the final electron acceptor in the cellular aerobic glucose catabolism and β-oxidation of fatty acids to efficiently generate ATP. Hence, oxygen deprivation (hypoxia) requires adaptive mechanisms to respond to this stress stimulus. Response to hypoxia is governed by the transcription factor hypoxia-inducible factor (HIF) that regulates the expression of at least 70 known target genes that allow the organism to adapt on the cellular, local and systemic level. HIF is a heterodimeric protein complex that consists of an oxygen-dependently regulated HIF-α subunit and a constitutively expressed HIF-β subunit. The HIF-α subunit protein stability is tightly regulated by hydroxylation of specific prolines in the oxygen-dependent degradation domain (ODDD) by prolyl-4-hydroxylases (PHDs) that require oxygen as a substrate for their enzymatic reaction. Hydroxylated HIF-α is targeted by the von Hippel-Lindau tumor suppressor protein (pVHL), the recognition component of an E3 ubiquitin ligase complex, and subsequently degraded in the 26S proteasome. Besides HIF-α, only a few other PHD hydroxylation targets have been described so far. We investigated the possibility of further hydroxylation targets for the PHD2 isoform and if other regulatory mechanisms apart from molecular oxygen might be important in the PHD2 protein regulation.

A yeast two-hybrid screening was carried out to identify novel PHD2 interacting partners. Among the identified interactors, we found the FK506-binding protein 38 (FKBP38). The specific interaction of FKBP38 with PHD2 but not PHD1 or PHD3 was confirmed in a variety of interaction assays, including yeast and mammalian two-hybrid analysis, GST pull-down and co-immunoprecipitation experiments and fluorescence resonance energy transfer (FRET) analysis. FKBP38 was shown to bind with a linear minimal glutamate rich binding motif from amino acid (aa) 37 to 56 to PHD2. Conversely, PHD2 interacts with its N-terminal region from aa 1 to 114, containing a MYND-type Zn^{2+} finger domain, with FKBP38.

Originally FKBP38 was identified as a target of the immunosuppressive drug FK506 and it has been proposed that FKBP38 regulates cell death or survival. FKBP38 belongs to the family of peptidyl prolyl *cis/trans* isomerases (PPIases) that participate in the conformational change of the aa proline from the *cis* to the *trans* position, providing the basis for a potential regulatory switch. Thus, we speculated that

FKBP38 might be a co-factor that is involved in the PHD-mediated enzymatic proline hydroxylation of HIF-α subunits.

Stable RNA interference (RNAi)-mediated downregulation of FKBP38 increased PHD2 protein abundance and cellular hydroxylation activity. Conversely, elevated PHD2 protein levels decreased hypoxic HIF-1α protein accumulation and attenuated HIF-dependent transcriptional regulation. Reconstitution of FKBP38 normalized PHD2 protein levels and therefore cellular hydroxylation capacity and HIF-1 response. Strikingly, increased PHD2 protein abundance was due to prolonged PHD2 protein stability, suggesting that FKBP38 determines PHD2 protein levels. However, we did not find any involvement of the FKBP38 enzymatic PPIase activity in the regulation of PHD2 protein abundance. So far, little is known about PHD2 protein regulation and we therefore further explored the molecular mechanism of FKBP38-dependent PHD2 protein regulation. PHD2 protein stability was neither influenced by a variety of protease inhibitors nor by inhibition of the ubiquitin-dependent proteasomal degradation pathway. Interestingly, in cellular assays the C-terminal transmembrane domain of FKBP38 is required for a specific interaction with PHD2 in spite of the existence of a functional FKBP38 interaction domain at the N-terminus. Consistent with these data, we could co-isolate FKBP38 and PHD2 from endoplasmatic reticulum and mitochondria membranes. Strikingly, regulation of PHD2 protein abundance requires the correct sub-cellular localization of FKBP38. As an outlook, targeting FKBP38:PHD2 protein interaction by chemical compounds might be an attractive strategy to specifically increase PHD2 amount and therefore attenuate HIF protein levels in tumors.

3 Zusammenfassung

Multizelluläre komplexe Organismen benötigen Sauerstoff als terminalen Elektronenakzeptor im zellulären Glukoseabbau und bei der β-Oxidation von Fettsäuren, um effizient ATP zu produzieren. Deshalb werden bei Sauerstoffmangel (Hypoxie) Anpassungsmechanismen benötigt, um auf diesen Stressstimulus adäquat zu antworten. Die Antwort auf Hypoxie wird durch den Transkriptionsfaktor Hypoxie-induzierbarer Faktor (HIF) gesteuert, der die Expression von mehr als 70 bekannten Zielgenen reguliert, die es dem Organismus ermöglichen, sich auf zellulärer, lokaler und systemischer Ebene anzupassen. HIF ist ein heterodimerer Proteinkomplex, der aus einer HIF-α Untereinheit besteht, die sauerstoffabhängig reguliert wird und aus einer konstitutiv exprimierten HIF-β Untereinheit. Die Proteinstabilität der HIF-α Untereinheit wird streng reguliert durch die Hydroxylierung von spezifischen Prolinen in der sauerstoffabhängigen Destabilisierungsdomäne (ODDD) durch Prolyl-4-Hydroxylasen, die molekularen Sauerstoff als Substrat für die enzymatische Reaktion essentiell benötigen. Hydroxyliertes HIF-α wird an das von Hippel-Lindau Tumor Suppressor Protein (pVHL) gebunden, das die Erkennungseinheit eines E3 Ubiquitin-Ligase Komplexes darstellt, und anschließend im 26S Proteasom abgebaut. Außer HIF-α sind gegenwärtig nur wenig andere Hydroxylierungssubstrate der PHDs bekannt. Wir untersuchten die Möglichkeit, ob weitere Hydroxylierungssubstrate für die PHD2 Isoform existieren und ob es neben Sauerstoff noch andere regulatorische Mechanismen gibt, die PHD2 regulieren.

Ein Zwei-Hybrid Screening in der Hefe wurde durchgeführt, um neue PHD2 interagierende Partner zu identifizieren. Unter den neu entdeckten Interaktoren fanden wir das FK506-bindende Protein 38 (FKBP38). Die spezifische Interaktion von FKBP38 mit PHD2 bestätigten wir in einer Vielzahl von Interaktionsstudien wie Hefe und Säugetier Zwei-Hybrid Interaktionsanalysen, in GST pull-down- und Co-Immunopräzipitationsexperimenten und in der Fluoreszenz Resonanz Energie Transfer (FRET) Analyse. FKBP38 bindet an ein lineares glutamatreiches minimales Bindungsmotiv von Aminosäure 37 bis 56 in PHD2. Umgekehrt interagiert PHD2 mit seiner N-terminalen Region von Aminosäure 1 bis 114 in FKBP38, die eine MYND ähnliche Zn^{2+} Fingerdomäne enthält.

FKBP38 entdeckte man ursprünglich als eine Zielstruktur für den immunsuppressiven Wirkstoff FK506 und es wird angenommen, dass FKBP38 den Zelltod oder das Überleben von Zellen reguliert. FKBP38 gehört zur Familie der Peptidylprolyl-

cis/trans-Isomerasen (PPIase), die die Konformationsänderung der Aminosäure Prolin von der *cis* in die *trans* Position steuern und kann die Basis für eine regulatorische Veränderung bilden.

Deshalb spekulierten wir, dass FKBP38 womöglich als Kofaktor fungiert, um die PHD gesteuerte enzymatische Prolinhydroxylierung der HIF-α Untereinheit zu regulieren. Jedoch erhöhte sich die PHD2 Proteinmenge und die zelluläre Hydroxylierungsaktivität durch die stabile Herunterregulation von FKBP38 durch RNA Interferenz (RNAi). Durch die erhöhte PHD2 Proteinmenge akkumulierte HIF-1α Protein in der Hypoxie weniger und die HIF-abhängige transkriptionelle Aktivität war abgeschwächt. Überraschenderweise war die Zunahme der PHD2 Proteinmenge aufgrund einer verlängerten PHD2 Proteinstabilität und deshalb schlagen wir vor, das FKBP38 die PHD2 Proteinmenge reguliert. Wir konnten bisher keine Beteiligung der FKBP38 PPIase Aktivitätsdomäne als Regulator der PHD2 Proteinmenge feststellen. Bisher ist sehr wenig über die PHD2 Proteinregulation bekannt und deshalb untersuchten wir den molekularen Mechanismus der FKBP38-abhängigen PHD2 Proteinregulierung. Die PHD2 Stabilität war weder durch eine Vielzahl von Proteaseninhibitoren beeinflusst noch durch die Blockierung des Ubiquitin-Proteasom Degradationssignalweges. Interessanterweise wird die C-terminale Transmembrandomäne von FKBP38 in zellulären Experimenten für die spezifische Interaktion mit PHD2 benötigt, obwohl die funktionsfähige Interaktionsdomäne am N-Terminus noch vorhanden ist. Konsistent mit diesen Daten konnten wir FKBP38 und PHD2 in endoplasmatischen und Mitochondrienmembranen isolieren und detektieren. Überraschenderweise benötigte die Regulation der PHD2 Menge die korrekte subzelluläre Lokalisation von FKBP38. Die Aufhebung der FKBP38:PHD2 Interaktion durch chemische Wirkstoffe könnte eine interessante Zielstruktur sein, um spezifisch durch die Zunahme von PHD2 die HIF Proteinmenge in Tumoren zu reduzieren.

4 Introduction

4.1 The hypoxia-inducible factor (HIF)

Reduced oxygen partial pressure (pO_2) greatly challenges human being at high altitude. The human body compensates a drop in barometric pressure by inducing a variety of physiological responses in order to maximally increase the delivery of oxygen as well as to optimize oxygen consumption within cells and tissues. Within minutes at arrival at high altitude the minute ventilation as well as cardiac output is increased. Later, in the process of acclimatization the number of red blood cells raises in the circulation due to stimulation of erythroid progenitor stem cells in the bone marrow by erythropoietin (EPO). Humans who constantly live above 3500 m in Bolivia or Tibet have roughly 10% higher hematocrit compared to residents at sea levels (1). But for a long time, it remained elusive what triggers the expression of EPO in the fetal liver and adult kidney in specialized peritubular fibroblasts within the cortex and outer medulla after exposure to low pO_2.

4.1.1 Discovery of the HIF complex

In 1991, a 50 nucleotide large *cis*-acting DNA element in the 3'flanking region of the human EPO gene was identified that mediates the hypoxic induction of a transiently transfected reporter gene plasmid in the hepatoma cell line Hep3B (2). A hypoxic binding activity was found to bind to the hypoxia responsive enhancer and its binding capacity was greatly enhanced upon hypoxia and sensitive to blocking translation by cycloheximide (CHX) (3). In 1995, Wang and Semenza succeeded to purify this hypoxic binding activity that turned out to be a heterodimeric protein complex consisting of a α-subunit (HIF-1α) and a β-subunit (HIF-1β) (4). HIF-1β also known as aryl hydrocarbon receptor nuclear translocator (ARNT) was already previously identified as heterodimerization partner of the dioxin receptor / aryl hydrocarbon receptor (AhR) and mediates xenobiotic responses (5).

Both proteins belong to the PAS family (period circadian protein (PER), ARNT and single-minded protein (SIM)) of basic helix-loop-helix (bHLH) transcription factors. So far three different α-subunits (HIF-1α, HIF-2α and HIF-3α) and one β-subunit (HIF-1β) are known.

4.1.2 Genetic ablation of HIF-α subunits

Genetic ablation of HIF-1α and HIF-2α showed non-redundant and different functions of both subunits during mouse embryonic development. Loss of HIF-1α led to developmental arrest and death at embryonic day (ED) E10.5 due to defects in neuronal tube formation, vascular and pharyngeal structures and cardiovascular malformation (6), (7). The vascular regression and abnormal remodeling of the vascular network was mainly due to HIF-1α dependent mesenchymal cell death rather than to the action of the vascular endothelial growth factor (VEGF) (8). Germline deletion of HIF-2α showed diverse phenotypes depending on the mouse strain used. In C57BL/6 mice, genetic ablation of HIF-2α resulted in embryonic death at mid-gestation. The mice suffered from pronounced bradycardia due to lowered production of catecholamine in the organ of Zuckerkandl resulting in improper cardiac function and circulatory failure (9). In ICR/129 sv genetic background, HIF-2α mice died because of defects in remodeling and formation of the vascular network (10). Furthermore, the deficiency of sufficient surfactant production by alveolar type 2 cells led to respiratory stress syndrome and subsequently death of neonatal mice (11). So far, no HIF-3α knockout mouse has been reported.

4.1.3 Expression pattern of HIF-α subunits

HIF-1α is ubiquitously expressed in different tissues and organs. In contrast to HIF-1α, HIF-2α and HIF-3α show a more restrictive expression pattern. HIF-2α also known as endothelial PAS protein 1 (EPAS-1) shares 48% identity with HIF-1α and its expression is highly detectable in embryonic and adult endothelium (12). Human HIF-3α has 6 alternative expressed splice variants (HIF-3α1 to 6). HIF-3α1 is proposed to be the full length protein and is mainly expressed in the kidney (13).

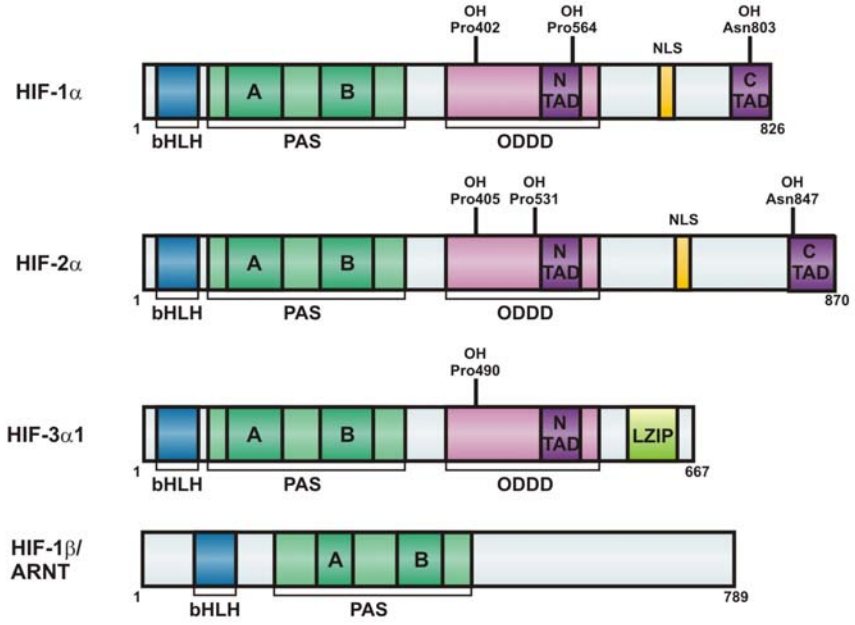

Figure 1. Domain architecture of HIF-α and HIF-1β

bHLH - basic helix-loop-helix domain; PAS - PER/ARNT/SIM; ODDD - oxygen-dependent degradation (ODD) domain; NTAD - N'terminal transactivation domain; CTAD – C-terminal transactivation domain; NLS - nuclear localization sequence; LZIP – leucine zipper domain

4.1.4 Composition of the HIF complex

HIF-α subunits contain a N-terminal basic helix-loop-helix (bHLH) DNA binding domain and a PAS domain that mediates HIF-1β dimerization (Fig. 1). Furthermore, HIF-α subunits possess two transactivation domains (TAD), a C-terminal TAD (CTAD) and a N-terminal TAD (NTAD) that overlaps with the oxygen-dependent degradation domain (ODDD) (Fig. 1). The ODDD is required for the stability of HIF-α subunits (14). Furthermore, a nuclear localization signal can be found. In contrast to HIF-1α and HIF-2α, HIF-3α1 does not contain a CTAD. Interestingly, most prominent expression of a HIF-3α splice variant (HIF-3α4) or known as inhibitory PAS (IPAS) protein is observed in mouse corneal epithelium. It might inhibit vessel formation to maintain an avascular phenotype in the cornea by binding to HIF-1α and therefore preventing HIF-1 complex formation and activation of HIF-1 induced target gene expression (15). Furthermore, the splice variant HIF-3α4 forms a complex with HIF-2α and interferes with the HIF-2 dependent hypoxia-induced gene expression and prevents tumor progression (16).

4.1.5 Oxygen-dependent regulation of HIF-α protein stability and transactivation activity

The protein levels of HIF-α subunits are tightly oxygen-dependently regulated and its half-live is less than 5 minutes. However, HIF-α mRNA levels are not affected. In normoxia, HIF-α subunits are rapidly degraded by the ubiquitin-proteasome pathway irrespective of its sub-cellular localization (17).

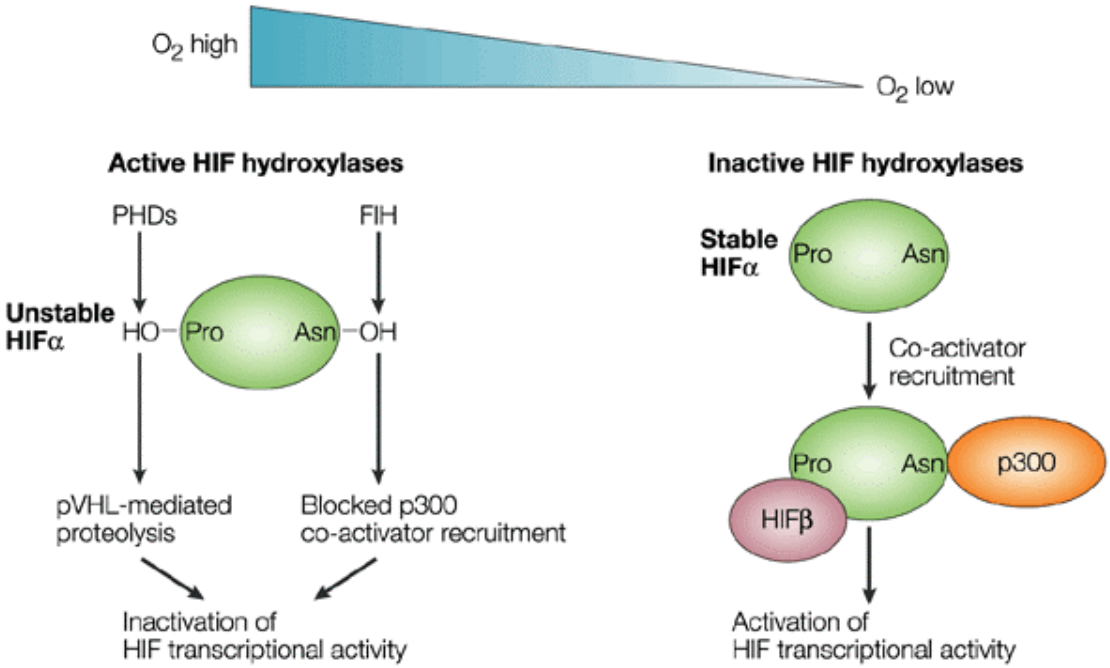

Figure 2. Oxygen sensing by HIF hydroxylases (18)

HIF-α subunits are regulated by PHDs and FIH via oxygen-dependent hydroxylation of conserved prolyl residues which leads to a pVHL mediated proteasomal degradation and inhibition of transcription of target genes by disrupting co-activator recruitment of the transcription initiation complex. Under reduced pO_2 HIF hydroxylase activity is decreased thereby leading to HIF-α stabilization and induction of expression of target genes.

A prerequisite for the ubiquitin-dependent degradation of HIF-α subunits by the 26S proteasome is the hydroxylation of specific prolines within the ODDD by oxygen-dependent prolyl-4-hydroxylases (PHDs) and subsequent binding of the von Hippel-Lindau (pVHL) tumor suppressor protein (19), (20), (21), (22). VHL is the recognition component of a multi-component E3 ubiquitin ligase (pVHL - elongin B - elongin C - Cul2 - Rbx) and targets HIF-α subunits for ubiquitinylation and rapid destruction by the 26S proteasome (Fig. 2). Loss of pVHL stabilizes HIF-α subunits under normoxia as manifest in vascular tumors like clear cell carcinoma, hemangioblastoma and pheochromocytoma (21). Additionally, the mouse acetyltransferase arrest-defective-1 (mARD1) has been suggested to acetylate amino acid (aa) 532 of HIF-1α, thereby enhancing the association with pVHL and accelerating HIF-1α degradation (23).

Contrary, other groups found no evidence for HIF-1α destabilization by acetylation (24), (25), (26).

Under hypoxia due to decreased PHD activity, HIF-α subunits are stabilized, translocate to the nucleus, heterodimerize with constitutively expressed HIF-1β, bind to hypoxia responsive element (HRE) and drive gene expression that mediates the adaptation to altered pO_2 (27). Furthermore, HIF transcriptional activity is regulated by factor inhibiting HIF (FIH) (28). FIH hydroxylates aa asparagine (N) 803 in HIF-1α and N847 in HIF-2α in the CTAD. Asparagine hydroxylation interferes with the binding of the cysteine/histidine rich (CH)-1 domain of p300/CBP and prevents transcriptional activation of the HIF complex (29). Michaelis Menten constant (K_m) values of O_2 are in the range between 230-250 μM for the prolyl-4-hydroxylases which correlates with the oxygen partial pressure and 90 μM for FIH (30), (31). Recently, lower K_m values of O_2 for the PHDs have been suggested depending on the length of the used HIF-derived substrate (32). This mechanism provides an elegant way to modulate oxygen-dependently HIF-α protein stability and transcriptional activity: Because PHDs are absolutely dependent on molecular oxygen, it provides a direct link between oxygen tension and HIF-α protein stability. Furthermore, oxygen-dependent full HIF complex activation is accomplished by a further drop in pO_2 leading to inactivation of FIH.

4.1.6 HIF complex function

HIF-α/β dimers bind to HREs and induce target gene expression. HREs consist of one or multiple minimal *cis* HIF binding sites (HBS) with the core sequence G/ACGTG (33).

So far, HIF is known to bind to about 70 HREs in genes and by microarray analysis it is suggested that there might exist more than 200 potential HIF target genes (33). In Figure 3 an overview about HIF target genes is given that are involved in the systemic, local and cellular response to hypoxia. HIF induces the expression of EPO that stimulates the production of red blood cells or the formation of new blood vessels by VEGF induction.

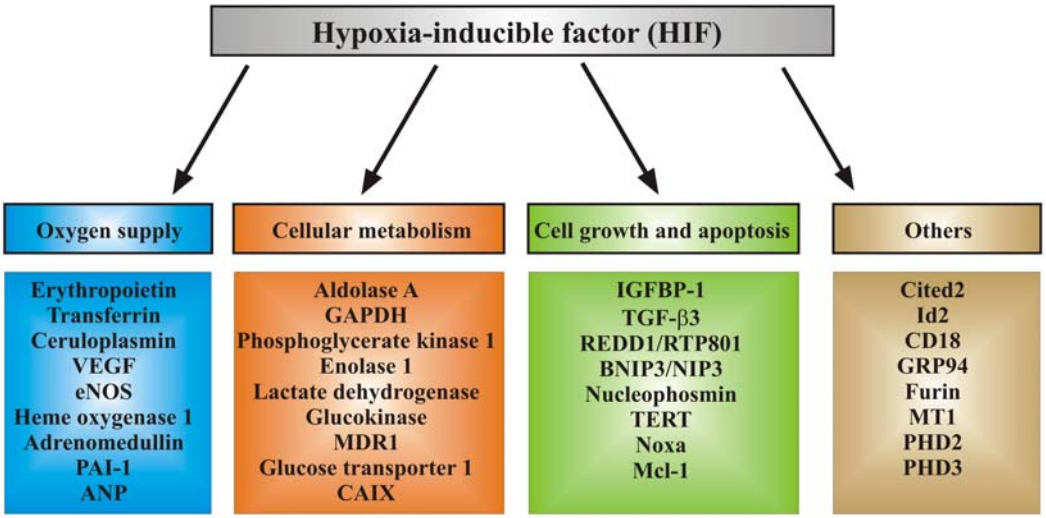

Figure 3. Putative induced target genes by HIF's (33)

ANP - atrial natriuretic peptide; BNIP3 - Bcl2/adenovirus E1B 19kDa interacting protein 3;CAIX - carbonic anhydrase 9; eNOS - endothelial nitric oxide synthase; GAPDH - glyceraldehyde-3-P-dehydrogenase; Id2 - inhibitor of DNA binding 2; GRP94 - 94 kDa glucose-regulated protein; IGFBP-1 - insulin like growth factor binding protein 1; Mcl-1 - myeloid cell leukaemia 1; MDR1 - multi drug resistance protein 1; MT1 - metallothionein 1; Noxa - phorbol-12-myristate-13-acetate-induced protein 1; PAI-1 - plasminogen-activator inhibitor 1; PHD2/3 - prolyl-4-hydroxylase 2/3; REDD1 - regulated in development and DNA damage response protein; TERT - telomerase reverse transcriptase; TGF-β3 - transforming growth factor β3; VEGF - vascular endothelial growth factor;

In addition, oxygen deprivation forces cells to change their cellular metabolism to anerobic glycolysis and lactate production to generate ATP. The HIF induced target gene pyruvate dehydrogenase kinase 1 (PDK1) phosphorylates pyruvate dehydrogenase (PDH) thereby actively suppressing the conversion of pyruvate to acetyl-CoA (34), (35). Therefore, pyruvate is exluded from mitochondrial consumption and PDK1 promotes the conversion of pyruvate to lactate by anerobic glycolysis.

Furthermore, cell growth and proliferation is altered in hypoxia. It has been shown that mammalian target of rapamycin (mTOR) function is regulated upon hypoxia by HIF-1. Under hypoxia (1.5% O_2) it has been published that mTOR as well as its targets are regulated to conserve energy (36). Essential for this inhibition of the mTOR pathway and therefore cell growth and proliferation, is a functional tuberous sclerosis (TSC) complex as well as the newly discovered protein regulated in development and DNA damage response (REDD1) (Fig. 10). Mouse embryonic fibroblasts (MEFs) derived from *Tsc*, *p53* double knockout embryos as well as *Redd1* deficient MEFs were not able to downregulate S6 kinase (S6K) under hypoxic conditions. Accumulation of REDD1 and RNA interference (RNAi)-mediated downregulation of tuberous sclerosis complex 2 (TSC2) tumor suppressor protein abolished this effect, placing REDD1 upstream of the TSC1/2 complex (37). Additionally, under growth supporting conditions TSC1/2 function is inactivated by

phosphorylation-dependent interaction with 14-3-3 proteins. In response to hypoxia, induced REDD1 expression by HIF-1 competes with TSC2 for 14-3-3 protein binding and subsequently stimulates TSC1/2 mediated mTOR pathway inhibition (38).

HIF-1 and HIF-2 complexes are closely related proteins and stimulate the expression of similar as well as unique target genes. HIF-1 appears to control glucose metabolism to induce a shift from aerobic to anerobic glycolysis like phosphoglycerate kinase (PGK), lactate dehydrogenase A (LDHA) and carbonic anhydrase 9 (CAIX) (39), (40), (41). HIF-2 might influence long-term hypoxic response and sustain mitochondrial homeostasis (42), (43). EPO, Oct4, Cyclin D and antioxidant enzymes Sod2 and Frataxin are suggested to be specific HIF-2 target genes (44), (45), (46), (47). Other target genes like VEGF, glucose transporter 1 (GLUT1) and NDRG1 are regulated by both proteins (48). So far, more research is required to discriminate between HIF-1 and HIF-2 dependent target gene expression.

HIF-1α and HIF-2α possess two TADs: NTAD and CTAD. NTAD shows low homology to CTAD (49). Recently, the different roles of the NTAD and CTAD in regard to differential HIF target gene expression have been investigated. Results suggest that the TCAD is the predominant domain that contributes to the transcriptional activation of HIF target genes. However, a subset of genes was exclusively dependent on NTAD (50). NTAD determines the HIF-1 and HIF-2 target specificity. The replacement of HIF-1α NTAD by HIF-2α NTAD was sufficient to convert HIF-1α to HIF-2α transactivation activity (51).

4.1.7 Regulation of HIF function

HIF is a target of posttranslational modification apart from hydroxylation. HIF-1α nitrosylation occurs at cysteine (C) 533 during tumor irradiation through generation of nitric oxide (NOS) by inducible NOS (iNOS) of activated macrophages in the tumor microenvironment (52). This posttranslational modification of HIF-1α resulted in HIF-1α protein accumulation and appears to be an alternative pathway independent of PHD-mediated HIF-1α degradation pathway. Nitrosylated HIF-1α ODDD does not bind to pVHL thereby enhancing HIF-1α accumulation and target gene expression. Additionally, nitrosylation of C800 of HIF-1α has been reported (53), (54). p300 binding to nitrosylated HIF-1α was decreased by quantitative interaction studies (53). Contrary, nitrosylation enhanced HIF-1 transcriptional activity as well (54).

HIF is described to be a phosphorylation target by the mitogen-activated protein kinase (MAPK) pathway. Initial studies demonstrated an *in vitro* HIF phosphorylation by p42/p44 MAPK, resulting in enhanced HIF transcriptional activity (55), (56). Phosphorylation of Serine (S) 641 and 643 by MAPK governs exportin 1 (CRM1) mediated nuclear translocation of HIF-1α and thereby controls HIF-1 transcriptional activity (57). Other reports suggested that p42/p44 MAPK is required for p300/CBP phosphorylation and subsequent binding to HIF-1α CTAD (58), (59).

In hypoxia, HIF-1α is modified by small ubiquitin-related modifier (SUMO) but this posttranslational modification and its functional consequence are a matter of debate. HIF-1α consists of 4 potential sumoylation sites but only lysine 391, 477 near the ODDD have been shown to be modified by SUMO 1 to 3 (60), (61). Recently, knockdown of Sentrin/SUMO-specific protease 1 (SENP1) in mice resulted in severe fetal anemia and embryonic death around day E13 to E15. These mice produced insufficient amount of EPO due to decreased stabilization of HIF-1α. Further analysis showed convincingly that sumoylated HIF-1α is VHL-dependently degraded by the proteasome and SENP1 could stabilize HIF-1α by removing SUMO from HIF. Therefore, hypoxic sumoylation might serve as a degradation signal in addition to proline hydroxylation for HIF-1α protein destruction even under hypoxic conditions. Contrary, other reports suggested that sumoylation stabilizes HIF in hypoxia and influences its protein stability and/or transcriptional activity (61), (60), (62).

Furthermore, HIF-1α protein levels might be dependent on its protein synthesis rate. It has been shown that a variety of growth factors, cytokines and mutated proteins involved in the mTOR signalling cascade, like loss of phosphatase and tensin homolog (PTEN) protein, induce HIF-1α protein synthesis due to increased translation (63), (64), (65), (66). Loss of promyelocytic leukaemia protein (PML) facilitates tumor angiogenesis by enhancing HIF-1α translation (67). Interestingly, under hypoxic conditions where mTOR activity and subsequently overall mRNA translation is reduced, HIF-1α mRNA however is still efficiently translated (68), (69). Originally, Lang et al. found an internal ribosome entry site (IRES) in the 5'untranslated region (UTR) of the mouse HIF-1α mRNA. This IRES allows an hypoxia-independent alternative mode of translational initiation and does not require eIF4E and a 5'cap (68). The polypyrimidine tract-binding protein (PTB) interacts with HIF-1α IRES and is enhanced upon hypoxia. PTB binding to HIF-1α mRNA IRES

participates in efficient translation in hypoxia (70). However, the widely used measurement of cellular 5'UTRs for IRES activity by dicistronic reporter plasmids has been questioned (71), (72), (73).

Additionally, the heat shock protein 90 (Hsp90) has been implicated in HIF regulation. Under normoxia, Hsp90 and Hsp70 are bound to the PAS B domain of HIF-α subunits and are replaced by ARNT upon hypoxia. Embryonic stem (ES) cells deficient for Hsp90 as well as treatment of the wild-type ES cells with geldanamycin delayed hypoxic HIF-1α accumulation (74). The receptor of activated protein C kinase 1 (RACK1) has been identified as a HIF-1α interaction partner. It binds to the PAS A domain of HIF-1α and competes with Hsp90 for binding. RACK1 depletion slightly stabilizes HIF-1α in normoxia and enhances HIF normoxic and hypoxic transcriptional activity. RACK1 mediated HIF-1α degradation is not influenced by oxygen but is dependent on functional 26S proteasome. RACK1 facilitates HIF-1α binding to elongin-C and therefore ubiquitinylation and degradation (75). RACK1-HIF-1α interaction is stabilized by spermidine/spermine-N-acetyltransferase-1 (SSAT1) (76). Interestingly, Hsp90 inhibitor 17-AAG, 17-DMAG and geldanamycin show a biphasic pattern in regard to HIF-accumulation and transcriptional activity. With low doses, the HIF transcriptional activity increased whereas under higher concentration HIF activity was lowered (77).

4.2 Prolyl-4-hydroxylases (PHDs)

4.2.1 Discovery and characterization of PHDs

It remained unknown how cells sense changes in oxygen partial pressure and subsequently regulate the stability of HIF-α subunits until quite recently. In 2001, by candidate gene approach in *C.elegans* a unique HIF prolyl-4-hydroxylase named egg-laying abnormal-9 (EGL-9) was discovered (78). In mammals, three HIF-prolyl hydroxylase domain containing proteins (PHDs) PHD1 (alternatively termed EGLN2, HPH3), PHD2 (EGLN1, HPH2) and PHD3 (EGLN3, HPH1) were identified (78), (79), (80). These enzymes are proposed to be the cellular oxygen sensors because of their ability to modulate HIF-α stability in dependency of the oxygen concentration.

Two PHD3 splice variants were described as a result of alternative splicing (81), (30). In *PHD3Δ4* exon 4 is deleted and results in 646 bp long mRNA. The 24 kDa PHD3Δ4 protein is still functionally active. *PHD3Δ1* has a large deletion in exon 1 and the expressed 13 kDa protein does not retain it's prolyl hydroxylase activity. Two splice variants have been reported for PHD2 lacking either exon 3 or 4 and led to inactive polypeptides (30). So far, no splice variant has been described for PHD1. However, a 34 aa shorter isoform (p40) of PHD1 is generated by alternative translational initiation with a molecular weight (MW) of 40 kDa which retains full hydroxylase activity (82). PHD1 p40 is less stable compared to the full length PHD1 with a MW of 43 kDa and like PHD1 not oxygen-dependently regulated. Recently, an endoplasmatic reticulum associated PHD proline 4-hydroxylase-related protein 4 (PH-4), was discovered (83), (84). This enzyme is able to hydroxylate both prolines in the ODDD of HIF-1α *in vitro* but the functional relevance of PH-4 in the oxygen-sensing pathway needs to be clarified.

4.2.2 PHDs are members of the 2-oxoglutarate-dependent family of dioxygenases

PHDs belong to the non-hem, Fe^{2+}- and 2-oxoglutarate-dependent family of dioxygenases. The catalytic domain consists of eight conserved antiparallel β-strands, which comprises the two β-sheets that constitute a jelly-roll-motif (85). The first known 2-oxoglutarate dioxygenase was the procollagen prolyl-hydroxylase that catalyses the prolyl hydroxylation of procollagen that is essential to stabilize the triple helix structure of collagen (86). Members of the 2-oxoglutarate-dependent

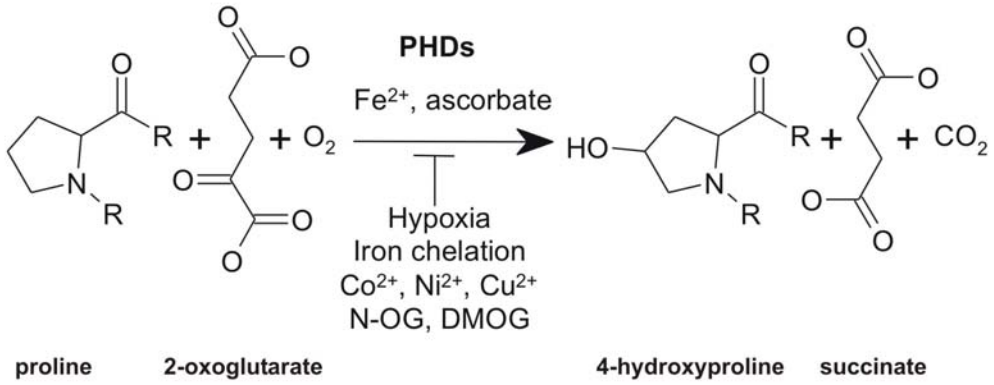

Figure 4. Proline hydroxylation catalyzed by HIF hydroxylases (87)

dioxygenases require di-oxygen, Fe^{2+} and 2-oxoglutarate for there enzymatic reaction.During catalysis, one oxygen atom is incorporated to a peptidyl proline (P) of the substrate to form a hydroxyproline and the other oxygen atom is used for a coupled decarboxylation reaction that converts 2-oxoglutarate into succinate and CO_2 (Fig. 4). Additionally, ascorbate is an essential component for a functional hydroxylase reaction and might protect the iron in the active center of the enzyme or the enzyme itself from oxidation by uncoupled reaction beside target hydroxylation (88), (89). Replacement of ascorbate by the antioxidant N-acetyl-L-cysteine did not restore PHD activity in *in vitro* VHL-dependent hydroxylation assays and emphasizes the important requirement of ascorbate for enzymatic activity (89).

PHD1 to 3 hydroxylate P564 of the ODDD of HIF-1α *in vitro* whereas P402 is only hydroxylated by PHD1 and PHD2 *in vitro* (78). Overexpression of each PHD in cellular systems suggests that all PHDs are active on both proline hydroxylation sites (90). At 20% oxygen P564 of HIF-1α was hydroxylated prior to P402 and hydroxylated P564 was required for P402 hydroxylation. PHD1 and PHD2 preferred to hydroxylate P564 based on its conformational structure whereas PHD3 favored P564 sequence independently of its localization. With decreasing pO_2 P402 hydroxylation was inhibited prior to P564 hydroxylation inhibition. Loss of P402 hydroxylation was already sufficient to stabilize HIF-1α at 0.5% oxygen whereas P564 was still hydroxylated (90).

PHDs are capable to hydroxylate HIF-1α peptides *in vitro* but with different specific activity. PHD2 and PHD3 from crude cell extracts from Sf9 cells display similar activity in a pVHL capture assay, PHD1 possesses less activity (91). However, in cellular systems PHDs are differentially expressed and therefore contribute differently to HIF-α hydroxylation depending on the enzyme abundance (92). Generally, the

hydroxylated prolyl residue for HIF-α resides in a strongly conserved LXXLAP motif (X stands for any amino acid) but only the hydroxylation target proline is absolutely mandatory for recognition (93), (94), (95).

4.2.3 Tissue distribution and sub-cellular localization of PHDs

PHD enzymes are differently expressed in tissues and organs. Using of monoclonal anti-PHD antibodies, a comprehensive overview about human PHD tissue distribution and sub-cellular localization has been done by Soilleux et al. (96). Most pronounced differences were observed in the heart with highest PHD3 expression and testis with strong PHD1 expression. PHD2 was widely found in most tissues (97), (83).

On sub-cellular level, fluorescent-fusion or tagged PHD1 protein was exclusively localized in the nucleus whereas PHD2 was found in the cytosol. PHD3 was equally detected in both compartments (98), (93).

In primary rat hepatocytes endogenous PHD1 was mainly found in the nucleus though at 1% O_2 PHD1 was bound to cytoplasm membrane and translocated back to the nucleus during reoxygenation (99). Endogenous PHD2 and PHD3 were described in the cytoplasm as well as in the nucleus. In hypoxia, PHD2 remained in the cytoplasm. Interestingly, some peroxisomal associated pattern was observed for all of the PHDs and additional mitochondrial staining for PHD2 and PHD3.

4.2.4 Genetic ablation of PHDs

Genetic disruption of the PHD2 gene led to embryonic lethality between embryonic days E12.5 and E14.5 whereas PHD1 and PHD3 knockout did not affect embryonic viability (100). *Phd2-/-* mice display developmental defects in the heart and the placenta leading to embryonic death. The cardiac function is disturbed as evident by enlarged intraventricular lumen, not fully developed trabeculae and closed interventricular septum. The placenta structure appeared to be irregulary formed by improper distribution of spongiotropoblast and giant cells. The labyrinth of highly vascularized villi of the placenta based on the fetus-derived blood vessels and the chorionic ectoderm was completely disorganized. Defects of placental development were a result of increased HIF-α protein levels. However, HIF-1α protein amount was not elevated in *Phd2-/-* mice hearts suggesting either a partial compensatory effect of PHD1 or 3 or PHD2 has other function apart from HIF-α hydroxylation (100). Global conditional inactivation of the PHD2 gene in adult mice resulted in mature and

perfusable blood vessels and increased vascular density in a variety of organs like ear, heart, liver, lung and kidney. HIF-1α accumulation led to increased expression of serum VEGF-A, but not elevated VEGF-A, angiopoietin 1 (ANG1) and ANG2 mRNA levels in various organs. Interestingly, despite inefficient disruption of Phd2 in brain, excessive vascular growth was observed and might be a result of systemic effects (101). As expected, somatic inactivation of PHD2 enhanced serum EPO levels and subsequently increased red blood cell production. Long term induced EPO protein caused polycythemia and as a result of it, hyperviscosity and cardiac dysfunction. Subsequently, these mice died from venous congestion and dilated cardiomyopathy (102), (103). Interestingly, HIF-1α protein but not HIF-2α accumulated in liver and kidney but only serum as well as kidney erythropoietin was increased. Heterozygous *Phd2* gene deletion did not facilitate erythrocytosis. Polycythemia did not arise from single *Phd1-/-* or *Phd3-/-* mice but double knockout mice showed induced elevated EPO levels by upregulating HIF-2α protein in the liver. Loss of *Phd1* gene shifted glucose metabolism from aerobic glucose oxidation to anerobic glycolysis thereby lowering oxygen consumption in skeletal muscle fibers. Genetic ablation of PHD1 enhanced PDK1 and PDK4 expression and therefore restricted the entry of pyruvate in the Krebs cycle. These mice showed a worse endurance performance. Muscle fibers were protected from ischemic necrosis after femoral artery ligation and loss of PHD1 induced hypoxic tolerance (104). Genetic ablation of PHD3 increased the number of superior cervical ganglion neurons in the sympathoadrenal system; however, the sympathoadrenal function seems to be disturbed and led to systemic low blood pressure (105).

4.2.5 Regulation of PHD expression and degradation

Proline hydroxylation is a non-reversible process and therefore PHDs itself are regulated on protein as well as on RNA levels. PHD2 and PHD3, but not PHD1 mRNA expression is oxygen-dependently regulated by HIF. PHD2 contains a functional HBS in the CpG island promoter region that is located approximately 3.5 kb upstream of the translation start codon (106). Interestingly, the hypoxia-responsive HBS of PHD3 is found in the first intron about 12 kb downstream of transcription initiation (107). Both proteins are direct HIF targets and induced under reduced oxygen tension (33). Enhanced hydroxylation activity due to increased PHD protein amount upon hypoxia accelerated HIF-1α protein degradation during reoxygenation

process (108), (109). Moreover, in long-term hypoxia increased PHD2 and PHD3 protein levels can compensate for reduced pO_2 and attenuate HIF-1α protein levels and it's target genes limiting the hypoxic response (110). Apart from oxygen, stimulation of serum-starved cells with transforming growth factor β (TGFβ) lowered PHD2 mRNA as well as protein amount and consequently prolonged HIF-1α protein stability (111). Oestrogen stimulation of breast cancer cell lines enhanced PHD1 mRNA (112), (92). Furthermore, PHD3 expression is modulated in different cell types by p53, by stimuli inducing smooth muscle cell differentiation and by nerve growth factor withdrawal (113), (114), (115), (116). Aging augmented specifically PHD3 protein in rat heart, skeletal muscle and liver and it could be countervailed by caloric restriction (117), (118).

PHDs display distinct mRNA and protein regulation as well as different degradation pathways. PHD1 and PHD3 have been reported to be targeted by E3 ubiquitin ligase Siah1/2 and subsequently proteasomal degraded (119). In *Siah1/2-/-* MEFs PHD3 protein stability is prolonged and resulted in decreased HIF-1α stability and HIF-dependent target gene expression. The lack of the N-terminal extension of PHD3 compared to PHD1 and PHD2 governs the susceptibility to Siah1/2-dependent ubiquitinylation and degradation (120). PHD2 is neither ubiquitinylated and degraded by the proteasome pathway (manuscript in preparation). PHD2 specifically interacts with the peptidyl prolyl *cis/trans* isomerase FK506-binding protein 38 (FKBP38) (121). Knockdown of FKBP38 by RNA interference (RNAi) increased PHD2 protein amount and prolonged its stability. PHD2 protein half life might depend on FKBP38 sub-cellular localization (manuscript in preparation).

4.2.6 Modulation of PHD activity

4.2.6.1 Oxygen

Oxygen is the limiting factor in the prolyl hydroxylase reaction. Therefore, availability of oxygen modulates the prolyl-4-hydroxylases. The K_m values of the three PHDs were determined by an *in vitro* hydroxylation-coupled decarboxylation assay. Radioactively labeled 2-oxo(1-^{14}C)glutarate was converted to succinate and $^{14}CO_2$ by using a 19-residue HIF-1α derived peptide as substrate. The K_m values for the three PHDs range between 230-250 μM and correspond to the K_m value for oxygen under normoxic conditions (30). Hence, changes in oxygen might easily limit prolyl hydroxylation and indicate the PHDs are effective oxygen sensors. However, K_m

values of the PHDs for O_2 decreases with increased peptide length and it is most likely that the K_m value might be lower than previously determined (32). Interestingly, tissue pO_2 is much lower (between 4-20 mm Hg) compared to 104-150 mm Hg in lungs. Clearly, the K_m value is still higher than tissue pO_2 and provides the basis for regulated PHD enzymatic reaction and HIF-α turnover (122). Furthermore, oxygen exchange of cells with its environment is not only governed by free diffusion but is also mediated by Aquaporin-1 (AQP-1) channels (123). AQP-1 overexpression accelerated HIF-1α accumulation and HIF target gene expression in hypoxia and downregulation of AQP-1 in normoxia promoted increased HIF-α levels. Thus, AQP-1 governs the flux of oxygen across the membranes depending on oxygen content in the environment thereby modulating the oxygen sensing pathway.

4.2.6.2 Energy metabolism intermediates

Prolyl-4-hydroxylase reaction requires tricarboxylic cycle (TCA) intermediates and limitation as well as excess of the Krebs cycle metabolites might affect PHD activity. Addition of oxaloaccetate, pyruvate and lactate promotes HIF-1α accumulation independently of oxygen in tumor cell lines (124), (125), (126). Enhanced HIF-1α protein levels were reversed by addition of ascorbate, cysteine, histidine and ferrous iron (126). Pyruvate and oxaloaccetate directly bind to the 2-oxoglutarate binding site of PHDs and thereby these enzymes were inhibited in normoxia (126), (127). Other groups did not find a pyruvate or oxaloaccetate dependent PHD inhibition *in vitro* or in cellular assays (127), (128). Furthermore, fumarate and succinate inhibit HIF-prolyl hydroxylases *in vitro* and prolonged HIF-1α half-life in cell culture models (129). Individuals with germline mutations in fumarate hydratase (FH) that catalyzes the conversion of fumarate to malate in the Krebs cycle are more prone to leiomyomas and renal cell cancer (130). Mice with conditional knockout of *Fh1* in the kidney developed renal cysts similar to human renal carcinomas (131). Mutations in the succinate dehydrogenase (SDH) are associated with high risk of developing paragangliomas and pheaochromocytomas (130). SDH converts succinate to fumarate. These tumors are characterized by accumulated HIF-1α and induced HIF target gene expression like VEGF leading to high microvessel density and tumor vascularization. Knockdown of SDH or FH by RNAi or succinate or fumarate addition in tumor cells stimulates HIF-1α accumulation and target gene expression by interfering with prolyl hydroxylase activity (132), (133), (129). Impaired PHD activity in

SDH-deficient cells was re-activated by treatment with cell permeating α-ketoglutarate derivatives and therefore restored HIF-1α impaired protein degradation (134). It is important to note that lack of SDH or FH might not only compromise PHDs but also other 2-oxoglutarate-dependent oxygenases in cellular systems (135).

4.2.6.3 Iron and divalent ions

PHDs belong to the family of Fe(II)- and 2-oxoglutarate-dependent dioxygenases that have a common jelly-roll motif (85). The jelly-roll motif possesses an iron-binding site in the active center. Purification of PHD2 to homogeneity co-purified endogenous iron and surprisingly zinc. Iron is mainly in its low spin form (Fe^{2+}) and only little ferric iron (Fe^{3+}) was observed (136). K_m values for iron for the three PHDs vary between 0.1 and 0.03 μM and its high affinity is consistent with strong iron binding to PHDs that is only slightly competed by the iron chelator desferrioxamine (DFX) and a variety of metals like Cd^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+} (137). But these compounds have been reported to have an impact on the HIF system. So it is most likely that other mechanisms in spite of competition exist to inactivate the HIF hydroxylases and thereby activating the HIF system. Interestingly, Cu^{2+} may attach to the active center and displace iron from its coordinated binding to histidine, aspartate/glutamate and histidine ligands (136).Therefore, it is able to stabilize HIF-1α under normoxia and increased reporter gene expression (138).

Furthermore, Ca^{2+} has been found to be involved in HIF pathway regulation. Cells treated with the specific Ca^{2+} chelator BAPTA, but not with Ca^{2+} ionophore A23187, HIF-1α was stabilized and accumulated in the nucleus (139). It interfered with PHD2 activity and addition of Fe^{2+} did not reverse the observed effect. Interestingly, rather changes in intracellular Ca^{2+} distribution than Ca^{2+} chelation contributed to PHD inhibition in an unknown manner.

4.2.6.4 Nitric oxide (NO)

Several groups suggested that nitric oxide derived from diverse NO donors or produced by iNOS provokes HIF-1α accumulation in normoxia (140), (141). NO-mediated HIF-1α stabilization is mainly caused by lowered PHD activity and might be mediated by formation of inhibitory nitrosothiols in the PHD protein, competing with oxygen binding or leading to oxidation at the iron in the catalytic center is oxidized (126). Furthermore changes in protein translation by NO-induced changes in PI3K

and MAPK signaling pathways might contribute to enhanced HIF-1α protein levels (142), (143). Contrary, in hypoxia the opposite effect is observed. Wang et al. suggested that the inhibitory effect of NO donors on HIF-1α protein accumulation might be due to changes in PHD activity in hypoxia (144). NO is a well known inhibitor of cytochrome *c* (Cyt *c*) oxidase (complex IV in the respiratory chain) and inhibits oxygen consumption by mitochondria. Therefore, oxygen is redistributed towards PHDs and enhances their activity even under reduced pO_2 and destabilizes HIF-α (145).

4.2.6.5 Reactive oxygen species (ROS) and antioxidants

Mitochondria have been implicated in regulating the oxygen sensing pathway. Inhibition of mitochondrial activity by mitochondrial inhibitors, deletion of mitochondrial DNA or genetic ablation of Cyt *c* impairs HIF-1α accumulation but the molecular mechanism is controversially discussed. Chandel et al. proposed that increased ROS under hypoxic conditions affected HIF-target gene expression (146). Complex III of the mitochondrial electron transfer chain (ETC) might serve as the ROS-generating protein complex (147), (148). However, different groups with different cellular models and conditions suggest different ROS levels and consequences for HIF-1α accumulation.

The impact of antioxidants to influence the oxygen signaling pathway might point towards a functional role of ROS in the HIF system. In tumors derived from c-myc dependent cells, tumorigenesis was reduced after treatment with N-acetylcysteine and ascorbate by decreasing HIF-1α levels. Reduction of tumor volume was dependent on functional PHD2 as well as pVHL (149). Furthermore, ascorbate is suggested to prevent oxidation of catalytic ferrous iron in prolyl hydroxylases (126) and ascorbate enhanced hydroxylation of a fusion protein containing of a GAL4 (β-galactosidase) DNA-binding domain, an HA (hemagglutinin Ha1 fragment) peptide and human HIF-1α ODDD in *vitro* thereby changing the mobility in SDS-PAGE analysis (128). Jun D is a transcription factor involved in oxidative stress defense and hydrogen peroxide H_2O_2 metabolism. Genetic ablation of Jun D impaired prolyl hydroxylase activity by increasing H_2O_2 and consequently iron oxidation to Fe^{3+} possibly through the Fenton reaction (150). Of note, Mucin 1 (MUC1) suppresses hypoxia induced accumulation of ROS, induces PHD3 expression and subsequently attenuates HIF transcriptional activity (151).

Another proposed hypothesis is the redistribution of oxygen after mitochondrial inhibition. Mitochondria are the main oxygen consuming organelles and inhibition of its function results in decrease in oxygen usage. Therefore, more oxygen is present in the cytosol and might activate prolyl hydroxylases even under hypoxia (145). Consistent with these data, mild hypoxia (3% O_2) prevented HIF-1α accumulation after mitochondrial inhibition in conventional dishes but not in gas-permable dishes where the oxygen gradient in the cell culture medium was eliminated. This effect was not observed under severe hypoxia (0.1% O_2) (152). Additionally, in PC12 AQP-1 overexpressing cells, HIF-2α was less stabilized after mitochondrial inhibition in hypoxia due to increased O_2 permeability (123).

4.2.7 Interactors and non-HIF-α hydroxylation substrates of PHDs

PHDs are not only regulated by its activity however interaction with other proteins might influence as well their function. We found a specific interaction of PHD1 with the onconeural antigen cerebellar degeneration-related protein 2 (Cdr2) (Kuppusamy et al., manuscript in preparation). Forced expression of Cdr2 reduced the expression of hypoxically induced target genes due to changes in the HIF transcriptional activity and HIF-1α protein accumulation.

PHD3 is suggested to be a target of the cytosolic chaperonin TCP1 ring complex polypeptide (TriC) (153). It is proposed that it plays a role in folding and specific complex formation because PHD3 tends to form aggregates (153), (154). The MAPK organizer 1 (Morg1) specifically interacts with PHD3 and supports PHD3 activity (155). Furthermore, it has been reported, that PHD3 is upregulated during C2C12 cell line skeletal muscle differentiation (156). Interestingly, downregulation of PHD3 by RNAi and antisense oligonucleotides decreased myogenin protein, a member of the MyoD family of myogenic regulatory factors that is involved in skeletal muscle differentiation. PHD3 interacted with myogenin and therefore facilitated myogenin stabilization. Association of myogenin with pVHL enhanced myogenin degradation. Thus, PHD3 might compete with pVHL for binding to myogenin and regulates myogenin protein stability.

Apart from HIF-α subunits, only a few potential PHD hydroxylation targets have been described so far. The nuclear factor-kappa B (NFκB) controls cell survival, proliferation and inflammation and is activated in hypoxia. In an inactive state, NFκB is bound to inhibitors of NFkB (IκB) and sequestered in the cytosol by masking the

nuclear localization sequence. Activation and translocation of NFkB to the nucleus is mediated by phosphorylation-dependent degradation of IκB by IκB kinases (IKKs). Downregulation of PHD1 or PHD2 by siRNA or DMOG treatment enhanced NFκB activity under normoxia whereas PHD1 overexpression decreased cytokine-stimulated NFκB activity. Interestingly, IKKβ expression is induced under hypoxia and it contains the conserved LXXLAP hydroxylation motif. Mutation of the proline within the LXXLAP motif in IKKβ yields in loss of NFκB activity inducibility. Hence, IKKβ stability might be determined by oxygen-dependent hydroxylation of PHD1 and therefore modulates NFkB activity under reduced oxygen tension (157).

The hyperphosphorylated carboxyl-terminal domain (CTD) of subunit 1 of RNA polymerase II (RP1) interacts with pVHL after UV radiation and DNA damage (158), (159). RP1 is targeted for rapid ubiquitinylation and destruction in the 26S proteasome. It is suggested that the interaction is hydroxylation-dependent and involves the PHDs.

PHD3 has been reported to modulate oxygen-dependently the activating transcription factor 4 (ATF-4) that is involved in cellular stress response (160). ATF-4 is transiently upregulated under severe hypoxia or stabilized after MG132 or DMOG treatment. PHD3 binds in the zipper II domain of ATF-4 and might modify prolines in this domain thereby regulating the half life of ATF-4 ubiquitin- but not pVHL-dependent. Until now, no PHD2 hydroxylation target apart from HIF-α has been characterized.

4.2.8 PHD's relevance in pathological disorders

A tumor is characterized by uncontrolled cell growth and proliferation. At a size of a few millimeters solid tumors suffer from deprivation of oxygen and nutrients. This results in hypoxic regions and upregulation of HIF and HIF-induced target gene expression thereby ensuring survival and growth of tumor cells (161). Ectopic expression of mPHD1 in HCT116 cells decreased in HIF-1 protein levels as well as target gene expression. Injection of mPHD1 expressing HCT116 cells in nude mice led to smaller tumor size, decreased microvessel density and more necrotic areas compared to animals injected with wild-type control cells (162). PHD2 expression in head and neck squamous cell carcinomas (HNSCC) is greatly enhanced compared to non-cancerous tissues and might be due to tumor hypoxia (163). Interestingly, enhanced PHD2 expression correlates with increased aggressiveness of tumors and nuclear translocation of PHD2. Pheochromocytomas, tumors derived from neural

crest cells, are characterized by germline mutations in *Sdh* or *Vhl* gene. Normally, in developmental processes of the sympathetic nervous system these cells die upon apoptosis after nerve growth factor (NGF) deprivation. PHD3 has been linked to promote apoptosis after NGF withdrawal and functions downstream of c-Jun in an unknown manner (164). Therefore, inactivation of SDH might limit PHD3 activity and subsequently foster neural crest cell survival and promotion of pheochromocytoma tumorous development.

PHD inhibition or activation might be an attractive tool to interfere with pathological features of disorders like stroke, anemia and ischemia. Administration of prolyl hydroxylase inhibitors increased EPO production by stabilization of HIF-α subunits and therefore increased red blood cell mass in rhesus macaques (165). However, long-term administration of PHD inhibitors in the treatment of anemia might promote tumorigenesis and angiogenesis in patients (166). Downregulation of PHD2 by RNAi in mice attenuated ischemia/reperfusion injury of the heart and decreased infarct size (167). PHD inhibition reduces cell death in neurons after transient global ischemia by bilateral carotid occlusion. Furthermore, in a permanent focal ischemia model where a filament is placed in the internal carotid artery to occlude the middle artery, administration of PHD inhibitors significantly reduced the infarct volume in the brain (168), (169).

The inflammatory bowel diseases are caused by an overactive immune system in the gastrointestinal tract. In murine models, colitis can be induced by administration of dextran-sodium sulphate (DSS) and the addition of the prolyl-4-hydroxylase inhibitor DMOG attenuated the progression of colitis and lowered the inflammatory markers IL-1β, TNF-α, IL-6 and Il-12 (170).

4.2.9 PHD2

Epstein et al. identified the *C.elegans* prolyl hydroxylase EGL-9 as the main enzyme that oxygen-dependently regulates the HIF-α subunits. By data base analysis the three human prolyl hydroxylases PHD1-3 were discovered. In spite of there ability to hydroxylate HIF-α subunits *in vitro*, they differ in there expression pattern, sub-cellular localization and tissue distribution. They might compensate each other and may have as well non-redundant functions.

PHD2 has been proposed to be the main oxygen sensor (171). Downregulation of PHD2 by RNAi was sufficient to stabilize HIF-1α under normoxia in HeLa cells.

However, different PHD expression levels in different cell lines do contribute differently to the regulation of HIF-α protein (92). PHD protein alignment revealed similar amino acid sequences in the C-terminal region that contains the catalytic domain but the N-terminal part greatly differs between the different isoforms.

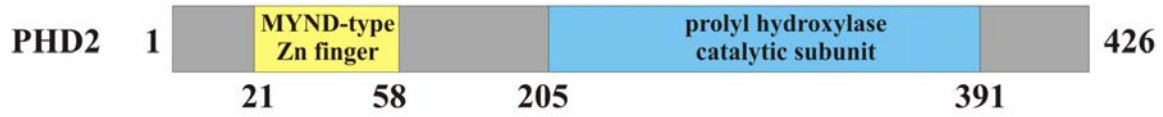

Figure 5. Domain structure of PHD2

PHD2 consists of a catalytic domain that consists of a prolyl hydroxylase catalytic subunit and a MYND (myeloid, nervy and DEAF-1) zinc finger domain (Fig. 5). MYND domain containing proteins are widely distributed among mammals, lower eukaryotic organisms and plants. MYND domain might function as a protein-protein interaction domain that provides the basis for negative transcriptional regulation. Deletion of the MYND-type zinc finger in PHD2 has been reported to enhance its activity and it is proposed that the PHD2 N-terminal region negatively modulates PHD2 catalytic activity (172). The zinc chelator N,N,N',N'-tetrakis (2-pyridylmethyl) ethylendiamine (TPEN) enhanced PHD2 hydroxylation ability but HIF-1α protein regulation was impaired due to inhibition of other Zn^{2+} containing proteins like CBP or Rbx (173).

Recently the crystal structure of PHD2 from aa 181 to aa 426 containing the catalytic subunit was described (174). PHD2, like other members of the family of 2-OG dioxygenases, has the typical double-stranded β-helix core fold. It crystallized as a homotrimer whereas it was found as a monomer in solution. The active site consists of a deep pocket between the major and minor β-sheets with a narrow opening.

Defects in the *Phd2* gene have been described to play a role in the development of polycythemia (erythrocytosis). Erythrocytosis is characterized by dramatically increased red blood cell mass due to genetic defects in erythroid progenitor cells or elevated EPO concentrations. A family was described that suffers from familiar erythrocytosis (175). Analysis revealed a C950G point mutation resulting in a amino acid exchange at position 317 from proline to arginine and complete loss of PHD2 hydroxylation activity. In addition, a novel PHD2 missense mutation (R371H) was reported (176). This mutation abolished HIF binding to PHD2 and increased HIF-dependent reporter gene activity. Interestingly, R371 mutation lies close to the

Pro317 mutation site and both sides may be part of the HIF-α substrate binding groove. Recently, also two frameshift mutations have been described (177). The base G at position 606 in exon was deleted resulting in a frameshift and a truncated 154 aa long C-terminal version of PHD2. Moreover, insertion of adenine between 840 and 841 bp created a frameshift and subsequently a truncated 143 aa PHD2 product. Heterozygous substitution of nucleotide 1129 caused a stop codon in the *Phd2* gene. So far, only a few proteins were reported to interact specifically with PHD2 and alter the oxygen signaling pathway. Amplified in osteosarcoma (OS9) protein was described as an essential complex component that promotes HIF-1α prolyl hydroxylation by PHD2 and PHD3 by interacting with both components at non-overlapping sites (178). Using yeast two-hybrid methology, the iron-only hydrogenase-like protein 1 (IOP1) was found to interact with PHD2. Downregulation of IOP1 enhanced HIF-1α protein amount and target gene expression by increasing HIF-1α mRNA levels however the role of PHD2 remained elusive (179). Furthermore, PHD2 was reported to bind to the tumor suppressor protein inhibitor of growth family member 4 (ING4) and might recruit it to HIF-1 complex and thereby modulating HIF transcriptional activity (180). Consistent with these data, PHD2 might interfere with its N-terminal transcriptional activity in hypoxia and limit HIF-mediated hypoxic response (181).

4.3 The peptidyl prolyl *cis/trans* isomerases (PPIases)

The superfamily of PPIases comprises the cyclophilins (Cyps), FK506-binding proteins (FKBPs), parvulins and the recently found protein serine/threonine phosphatase 2A (PP2A). Cyps and FKBPs were previously identified as cellular receptors for the clinically used immunosupressive drugs cyclosporin A (CsA), FK506 (tacrolimus) and rapamycin (sirolimus). These compounds greatly improved the treatment of graft rejection after organ transplantation and autoimmune diseases. They suppress the maturation and proliferation of native T-cells into effector cells. T-cell activation occurs after binding of specific antigen associated with the major histocompatibility complex (MHC) to the T-cell receptor. Ligand-dependent activation of T-cell receptor activates a signal cascade resulting in cellular Ca^{2+} increase. Ca^{2+} activated calmodulin (CaM) interferes with the serine/threonine phosphatase calcineurin and dephosphorylates nuclear factor of activated T-cells (NFAT). Dephosphorylated NFAT translocates to the nucleus and induces NFAT-dependent target gene expression e.g. interleukin 2. Secreted interleukin 2 binds to its receptor on T-cells and activates the mTOR pathway to induce cell growth as well as proliferation. CsA forms a ternary complex with cyclophilin A (CypA) and FK506 associates with FK506-binding protein 12 (FKBP12). Both complexes block calcineurin phosphatase activity (182). Furthermore, rapamycin binds to FKBP12 and suppresses mTOR kinase activity (183).

Although structurally unrelated in their amino acid sequences, all members of the protein family of PPIases exhibit a potential PPIase activity and can be inhibited by different types of immunosuppressive compounds. The catalytic domain contains a central β-sheet (184). Interestingly, FK506, rapamycin and CsA do impair PPIase-enzymatic activity *in vitro* due to binding in the catalytic pocket of the PPIase domain but the immunosuppressive action is rather due to the formation of a ternary complex with calcineurin or mTOR, respectively (185), (186), (187).

The amino acid proline exists in distinct *cis* or *trans* conformations within a protein structure and provides the basis for a potential regulatory switch (Fig. 6). Though the *trans* state (torsion angle = 180º) is energetically more favorable than the *cis* conformation (torsion angle = 0 º), imide peptide bonds (X-Pro) are found only in 5 to 6% of all protein structures in *cis* conformation (188), (189).

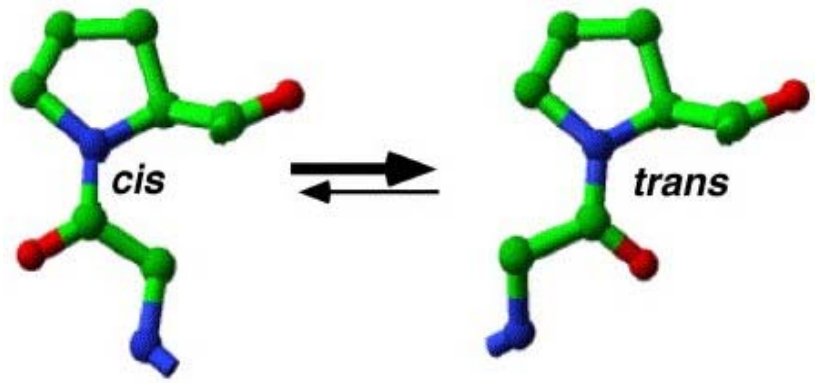

Figure 6. Prolyl cis/trans isomerization (190)

PPIases enzymes catalyze the *cis /trans* isomerization of prolyl bonds between *cis* and *trans* conformation.

In fact, prolyl *cis/trans* isomerization process is a very slow reaction as a result of high-energy barrier in conjunction with torsion angle of 90º and is greatly accelerated by PPIase catalysis (191). However, the precise mechanism remains to be solved. PPIases are thought to be involved in protein folding but growing evidence supports their role in regulating cellular processes like cell cycle, signaling regulation and gene expression.

Furthermore, PPIases are suggested to have a chaperone function distinct from the PPIase enzymatic activity (192), (193). Chaperones facilitate folding and unfolding as well as assembly and disassembly of macromolecular complexes and attenuate protein aggregation. The chaperone activity of FKBP51/52 was mapped *in vitro* in the C-terminal region including the tetratricopeptide repeats (TPR) that are well known for protein-protein interaction like Hsp90 and separates it from the PPIase domain (194). On the other hand, the chaperone function of cyclophilin 40 (Cyp40) was *in vitro* located in the linker region between PPIase domain and TPRs. Hence, the domain or required residues containing the chaperone activity remains to be identified.

Cyclophilin prototype cyclophilin A (Cyp18) was first discovered by its high affinity to cyclosporin A (195). Subsequently, further cyclophilins were identified by its cyclosporine A binding ability. So far, 7 major cyclophilins are known in humans: CypA, B, C, D, E, 40, NK (196). Beside a common PPIase activity, they greatly differ in there sub-cellular localization and potential function. Cyclophilin A, 40 and NK are localized in the cytosol and function in the maturation of neuronal receptors as well as steroid-receptor complex formation. Cyclophilin B and C are endoplasmatic reticulum secretory pathway associated proteins (197), (198). Cyclophilin D protein is part of the mitochondrial permeability transition pore (MPTP) together with the

voltage-dependent anion transporter (VDAC) and adenine nucleotide translocase (ANT) (199). Cyclophilin D might be inducing a conformal change in ANT by oxidative stress and Ca^{2+} overload thereby opening the pore and stimulating rather necrotic cell death than apoptosis (200), (201), (202).

FKBP12 was isolated by it's binding capability to FK506 in the cytosol of T-Jurkat cells (203). It belongs to a phylogenetic widely distributed family. New members were discovered by sequence homology comparison. So far, 7 human FKBPs are known which differ in size, structure, distribution and function (204). FKBP12, 13 and 25 are single domain proteins that function in receptor regulation and attached to erythrocyte membranes or nuclear proteins like casein II and nucleolin. The multi-domain proteins like FKBP51 and 52 are required in the formation of Hsp90 associated progesterone or steroid receptor complexes (205), (206).

The most prominent and unique member of the parvulin family is Pin1. Neither FK506 nor CsA interfere with the peptidyl prolyl *cis/trans* isomerase activity and Pin1 specifically recognizes phosphorylated Pro-directed Ser/Thr (pSer/Thr-Pro) peptide sequences (207), (208). Phosphorylation of p53 after DNA damage enables Pin1 to interact with p53 thereby preventing interaction with Mdm2 and subsequent p53 proteasomal destruction (209), (210). Hence, Pin1 enhances p53 stabilization and p53 target gene expression. Furthermore, Pin1 is required in proper processing of the amyloid precursor protein (APP) (211). Phosphorylated APP phosphorylation tends to be in *cis* conformation that forwards APP to the amyloidogenic processing pathway via β- and γ-secretase and results in insoluble Aβ42 peptides. Pin 1 converts APP in *trans* position thereby APP processing occurs via α- and γ-secretase pathway resulting in non-toxic APP fragments.

4.4 FKBP38

4.4.1 Discovery and characterization of FKBP38

FK506-binding protein 38 (FKBP38) is a member of the FKBP family that binds the immunosuppressive drug FK506. First, FKBP38 was described by Elsa Lam et al. in 1995. They screened for cDNAs from Jurkat cells that contain the DNA sequence for the well conserved second β-sheet region of FKBP12 from amino acid 26 to 34 to identify novel FKBP homologs (212). A cDNA was found that encodes a 38 kDa protein, FKBP-related 38 kDa protein (FKBPr38) that is 33% identical to FKBP12. As a matter of fact, the 38 kDa form resulted from a truncated open reading frame. Nielsen at al. 2004 described a 47 amino acid encoded extended open reading frame of FKBP38 and named it FKBP8 (213), (214). It encodes for a 45 kDa protein in human and 44 and 46 kDa protein splice variants in mouse but no further known domains, signal sequences as well as glycosylation sites were discovered.

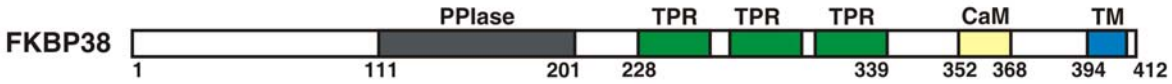

Figure 7. Domain structure of FKBP38

PPIase - peptidyl prolyl *cis/trans* isomerase activity domain; TPR - tetratricopeptide repeat domain; CaM - calmodulin binding domain; TM - transmembrane domain

The human FKBP38 (=FKBP8) gene is located on chromosome 19p12. FKBP38 possesses a PPIase domain, three 34 amino acid long tetratricopeptide repeat domains (TPRs) similar to FKBP52, a CaM binding site and transmembrane domain (TM) at the C-terminus (Fig. 7). The second TPR overlaps with a consensus leucine zipper motif (212), (213).

Highest levels of FKBP38 human mRNA expression are observed in the brain and moderate expression can be found in the heart, lung, skeletal muscle and pancreas and low expression in placenta and liver. In mice, FKBP38 is widely expressed in mouse brain tissues including neurons of the forebrain, striatum and thalamus and cells of Purkinje cell layer of the cerebellum. Transgenic mice expressing lacZ in front of a putative 4.4 kb FKBP38 promoter region showed an abundant expression in hippocampus, neocortex, striatum and cerebellum. NF-Y and SP1 binding sites were found in the promoter region of FKBP38 (213). On the protein level, FKBP38 is widely expressed in murine embryonic and adult tissues as well as in human cancer cell lines (215), (216). FKBP38 is an integral membrane protein located in

mitochondria and ER membranes exposed to the cytosolic face. Furthermore, FKBP38 is suggested to be posttranslationally modified but not by glycosylation (215).

Amino acid sequence analysis of the FKBP38 PPIase domain showed strong differences compared to other PPIases. It lacks the conserved amino acid tryptophan (W) at position 59 and has instead a Leucin (L). Tryptophan forms the basis of the hydrophobic FK506 drug-binding cavity that comprises the PPIase domain. Therefore, it was suggested that FKBP38 does not contain a PPIase activity (212). FKBP38 alone does not exhibit an enzymatic activity in an *in vitro* protease-coupled *cis/trans* isomerase activity assay. However, it absolutely requires Ca^{2+} and CaM as co-factors to function as an active enzyme (217). Inhibition of complex formation with CaM inhibitory myosine light chain kinases (MLCK) peptides, FKBP38 activity was completely abolished (217). CaM possesses two globular domains connected with a linker. The C-terminal part from aa 76 to 148 binds calcium-dependently to the CaM binding site in FKBP38 whereas the N-terminal aa 1 to 75 link the N-terminal region of FKBP38 (aa 35-153) to a so far unknown CaM binding site in a Ca^{2+} independent manner (218).

Genetic disruption of FKBP38 by deletion of FKBP38 exon 4-6 resulted in embryonic lethality at embryonic day E13.5 (215). Detailed analysis showed defects in the development of the central nervous system. The retina and pigmented epithelium was reduced and this results in failure of eye development. Ventral cell fates were remarkably increased on the expense of the dorsal fates. This phenotype seems to be similar to constitutively active sonic hedgehog signaling (SHH) pathway or inhibition of the SHH antagonist protein kinase A (PKA) (219). Indeed, in *Fkbp38-/-* mice the SHH pathway is constitutively activated. The SHH signaling pathway is crucial in embryonic pattering and cell fate determination. De-regulation of this signaling pathway supports tumor growth in skin, muscle and brain (220). As a secreted morphogen, SHH binds to its receptor patched (Ptch1) and suppresses the inhibitory effect of Ptch1 on Smoothened (Smo) (Fig. 8). Through a so far not completely understood signal cascade, the transcription factors Gli are not proteasomally cleaved and induce target gene expression.

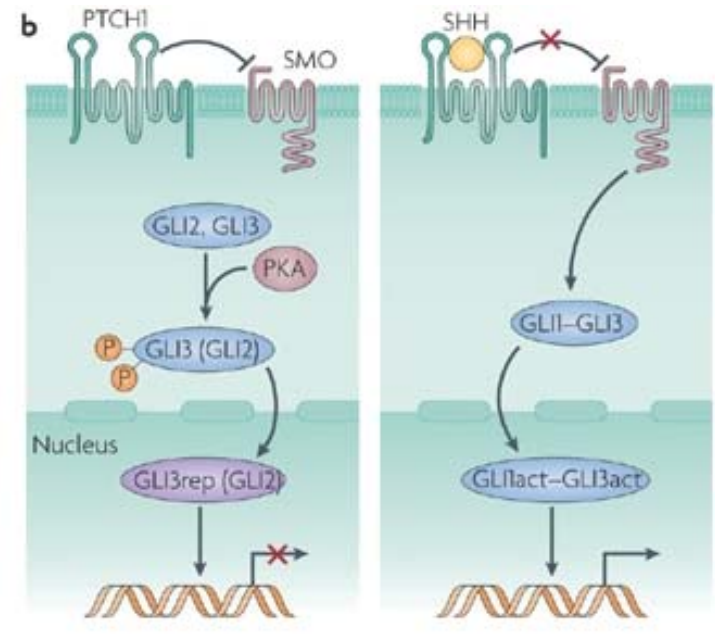

Figure 8. Sonic hedegehog signaling pathway (221)

In the absence of SHH receptor binding, Gli transcription factors are processed in the proteasome and the carboxy-terminally truncated version suppresses Gli-mediated target gene expression. So, genetic ablation of FKBP38 resulted in induced expression of SHH signaling target genes like Ptch1 and Gli transcription factors. Double knockout of FKBP38 and SHH rescued the FKBP38 knockout phenotype. Therefore, FKBP38 may negatively regulate, most likely ligand-independently, the SHH signaling pathway during development of the central nervous system.

Mice with homozygous disruption of exon 3 to 7 of *Fkbp38* completed embryonic development however the mice died soon after birth (222). These mice suffered from defects in the closure in thoracico-lumbo-scaral region (spina bifida) and skeletal defects. Neural tube defects were furthermore observed in FKBP38 knockout mice generated by gene trap insertion (223).

4.4.2 Physiological functions of FKBP38

4.4.2.1 Impact of FKBP38 on calcineurin activity

The best characterized FKBP member, FKBP12, forms a complex with FK506 and the FKBP12-FK506 complex interferes with calcineurin and its interaction with its substrates like NFAT, MEF2, Elk-1, NOS and PKA. FK506 binds in the PPIase domain and mimics endogenous ligands to block calcineurin phosphatase activity. Therefore, Shirane et al. proposed an intrinsic inhibitory effect of FKBP38 on calcineurin (224). Tagged isolated FKBP38 from HeLa cells reduced the dephosphorylation capacity of calcineurin on synthetic phosphopeptide of RII subunit of PKA. Increased Ca^{2+} influx by A23187 decreased phosphorylated NFAT4, a known calcineurin target and overexpressed FKBP38 lowered dephosphorylation of NFAT4. Furthermore, expression pattern of FKBP38 in mouse brain resembles that

of calcineurin (213). Contrary to Shirane et al., other groups did not observe neither an inhibitory effect on calcineurin phosphatase activity by FKBP38 *in vitro* nor changes in the NFAT-responsive reporter gene activity upon FKBP38 overexpression in SH-SY5Y cells and Jurkat cells (225), (216). Furthermore, FKBP38 does not physically interact with calcineurin and it does not influence the PPIase activity of FKBP38. Only, the complex of FKBP38 and FK506 inhibits calcineurin activity but to a much lesser extend than FKBP12-FK506 complex (226).

4.4.2.2 Functional role of FKBP38 in apoptosis

FKBP38 association with the anti-apoptotic protein Bcl-2 might have a functional role in apoptosis process but it is a matter of intense debate. Programmed cell death is essential to eliminate aged, injured or infected cells and supports correct development of multicellular organisms. In apoptotic cells, the DNA condensates and is degraded, the membrane starts to bleb and the cell breaks apart in vesicles that are absorbed by phagocytes without inducing an inflammatory response.

Self destruction is induced by ligand-dependent binding to the death receptors of the tumor necrosis factor (TNF) family. This extrinsic pathway mediates the activation of caspase-8 (and caspase-10 in humans) and downstream caspases that provoke cell destruction. Independently, programmed cell death is also induced by diverse intracellular stresses including growth factor or cytokine deprivation, accumulation of unfolded proteins or genotoxic damage and triggers the release of Cyt *c* from mitochondria. Subsequently, Cyt *c* forms a complex with apoptotic protease activating factor-1 (Apaf-1) that activates the initiator caspase 9 and later on the executioner caspases 3, 6, 7 (Fig. 9). This pathway is mainly governed by the Bcl-2 family. The Bcl-2 family comprises the pro-survival proteins like Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, the cell death promoters Bax-like apoptotic proteins Bax, Bad, Bak and the BH3-only proteins Bik, Bid, Bim, Noxa and Puma (227). The current model suggests that the pro-survival proteins engage with the anti- apoptotic proteins and prevent disturbance of mitochondrial membrane structure by pore formation in the outer mitochondrial membranes (228).

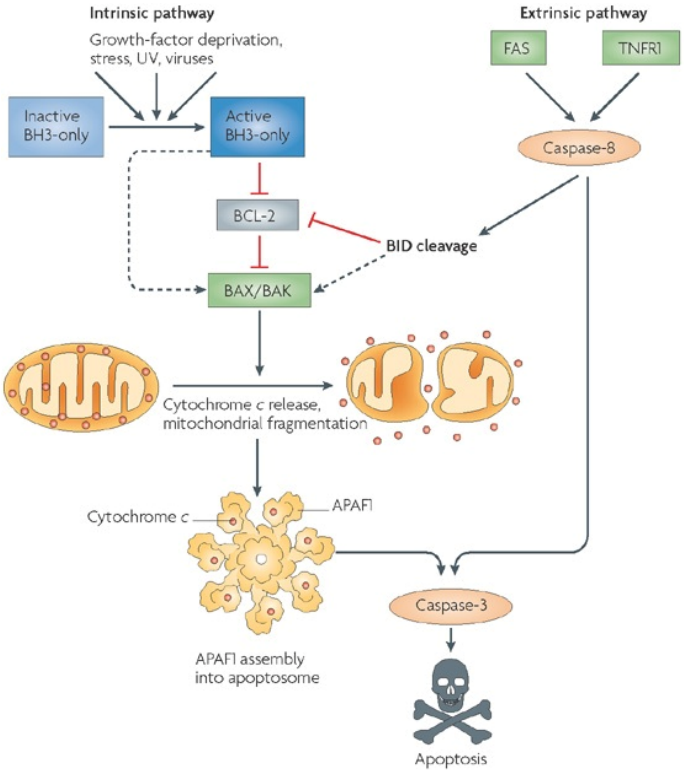

Figure 9. Apoptosis pathway (229)

Intracellular stress and developmental cues trigger the BH3-only proteins as cell damage sensors to interact with the pro-survival proteins in the hydrophobic grove of the globular BH1, 2, 3 domains and promote the translocation of pro-apoptotic proteins to mitochondria to induce Cyt *c* release. In HeLa cells, FKBP38 targets Bcl-2 as well as Bcl-x_L to mitochondrial membranes thereby facilitating Bcl-2 and Bcl-x_L in their anti-apoptotic role (224). FKBP38 downregulation by RNAi strongly reduced HeLa cell viability and greatly enhanced caspase-3 activity as well as PARP cleavage, a caspase-3 substrate (216). Protein stability of FKBP38 and Bcl-2 is determined by Presenilin 1/2 where mutations cause early onset of familiar Alzheimer`s disease (FAD). Genetic ablation of Presenilin 1/2 increases FKBP38 and Bcl-2 protein abundance. So, cells were less susceptible to intrinsic apopotic stimuli due to increased Bcl-2 translocation to mitochondria (230).

Additionally, Edlich et al. reported a Ca^{2+}-CaM dependent interaction of FKBP38 with Bcl-2 that is blocked by Hsp90 binding to FKBP38. However, they propose that FKBP38 promotes cell death in SH-SY5Y neuroblastoma cells. Blockage of FKBP38 by the FKBP inhibitor GPI1046 lowered the decrease in cell viability after induction of cell death by diverse inducers like etoposide, camptothecin and ionomycin and can be reversed by overexpression of Hsp90 in this cell line (217), (231). Furthermore, FKBP38 inhibitor DM-CHX reduced neuronal cell death in rats subjected to transient focal cerebral ischemia suggesting its pro-apoptotic function.(226). Interestingly, overexpression of FKBP38 reduced matrix metalloproteinase 9 (MMP9) and upregulated syndecan 1 (SDC1), proteins involved in invasion and metastasis of tumors suggesting anti-metastatic and anti-invasive functions (232).

4.4.2.3 FKBP38 as a chaperone

FKBP38 may also function as a chaperone. Recombinant FKBP38 was able to reduce thermal denaturated citrate synthase aggregates *in vitro*, a chaperone activity measurement (216). Global cystic fibrosis transmembrane conductance regulator (CFTR) interactome protein analysis demonstrated FKBP38 as a co-chaperone. Cystic fibrosis is an inherited disease that results in the defective folding and export of the CFTR protein from ER due to deletion of aa F508. Expressed CFTR is expressed in apical epithelial cells like lung, liver, pancreas, skin and functions as a cAMP-regulated chloride channel that moves chloride ions to the covering mucus and regulates the fluidity of mucus and secretions. Patients with cystic fibrosis suffer from airway obstruction by thick mucus and chronic infection which eventually results in loss of pulmonary function (233).

FKBP38 affects the stability of CFTR and seems to be tightly associated to the steady-state levels of Hsp90 to support the Hsp90-client pathway (234). Indeed, FKBP38 itself interacts with the chaperone Hsp90 (235). Furthermore, FKBP38 associates with HERG, a voltage-dependent potassium channel supports the full maturation of HERG (236) as well as with Protrudin, a modulator of neurite formation and membrane trafficking (237).

4.4.2.4 FKBP38 as an organelle linker

FKBP38 has been reported to associate to the S4 subunit of the 19S proteasome via its TPRs (238). The 19S proteasome is the regulatory particle and forms with the proteolytic core (20S proteasome) the 26S proteasome.

The 26S proteasome is an ATP-dependent macromolecular complex and regulates protein stability by its ubiquitin-dependent degradation. In primary FKBP38 knockout cells the 26S proteasome is more localized in the cytosol then to membrane fractions and a decrease in proteasomal activity was observed in the membrane compared to cytosolic fraction. Therefore, FKBP38 might link the proteasome to mitochondria as well as ER to increase proteasomal activity at these organelles.

4.4.2.5 FKBP38 and its role in the mTOR pathway

mTOR is the master regulator of cell growth, size and proliferation and integrates diverse signals e.g. nutrients, energy state and growth factors (Fig. 10).

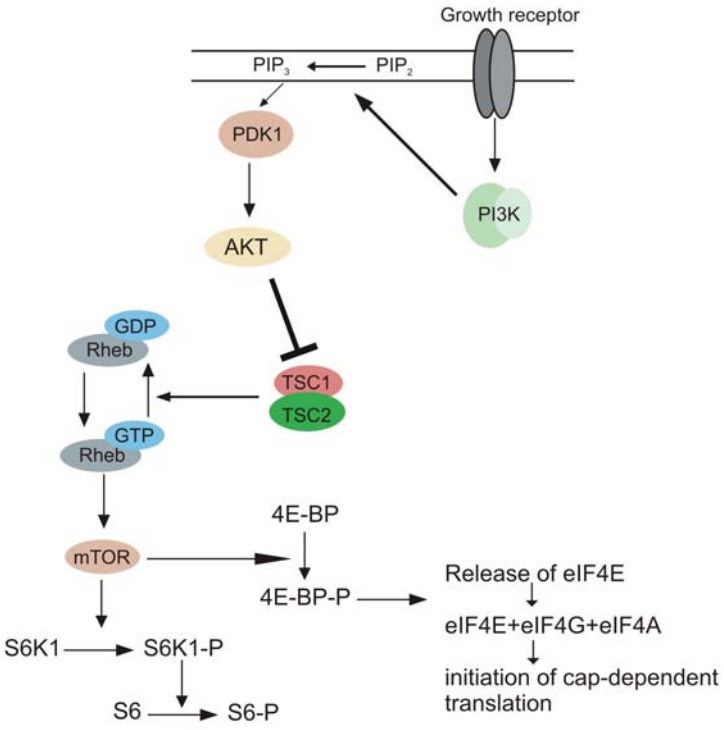

Figure 10. The mTOR signaling pathway

Under growth conditions, the mTOR pathway is stimulated by diverse growth factors like insulin. Over a cascade of adaptor proteins the phosphoinositide-3 kinase (PI3K) is activated and phosphorylates PIP_2 into PIP_3. Only PIP_3 is able to recruit PDK1 and AKT to the cell membrane. Activation of PDK1 leads to activation of AKT. AKT is a serine/threonine kinase that blocks the activity of the TSC complex. The TSC complex is composed of the tumor suppressor proteins hamartin (TSC1) and tuberin (TSC2) that mediate the inhibition of mTOR by acting as a GTPase-activating (GAP) protein stimulating the GTPase activity of the ras homolog enriched in brain (Rheb) protein. In its active state, the GTP-bound form stimulates the serine/threonine kinase activity of mTOR. Activated mTOR phosphorylates the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (E4-BP1) that releases eIF4E which then increases cap-dependent translation. The p70 ribosomal protein S6 kinase 1 (S6K1) is phosphorylated by mTOR, activates the 40S ribosomal protein S6 and enhances the translation of mRNA's that contain repressive 5'-terminal oligopyrimidine (5'-TOP) tracts. These mRNAs encode elongation factors as well as ribosomal proteins reviewed by Li et al. (239). Under conditions that are adverse for proliferation, this pathway is inactivated by TSC1/2 whereas the TSC1/2 complex is blocked by AKT under growth favourable conditions. TSC1/2 overexpression slightly reduced the cell size but this effect was reversed by FKBP38 downregulation with antisense olignucleotides (240). Furthermore, FKBP38 mRNA levels were upregulated upon TSC1 overexpression. Recently, FKBP38 has been reported to compete with Rheb for mTOR binding (241). When sufficient nutrients and growth factors were present, GTP-Rheb accumulated and prevented FKBP38 binding to mTOR and activated the phosphorylation of mTOR targets S6K and 4E-BP. Nutrient and growth factor

deprivation inactivated mTOR by enhanced FKBP38 binding. Therefore, FKBP38 might function as an endogenous mTOR inhibitor by interfering with Rheb and modulating cell growth and size.

In summary, accumulating data implicate a multifunctional role of FKBP38 in cell regulatory processes like cell signaling, apoptosis, cell proliferation and growth.

4.5 Working hypothesis

Prolyl-4-hydroxylases are the key enzymes regulating the stability of the HIF-α subunits. PHD activity is attenuated by the availability of the substrate O_2 but also by other factors like ROS, divalent cations, antioxidants or Krebs cycle intermediates. Due to the fact that there are at least three PHD isoforms capable of hydroxylating HIF-α subunits *in vitro* and differing in their tissue distribution and sub-cellular localization and *in vivo* function as determined by genetic knockout models, we hypothesize that PHDs have redundant as well as non-redundant functions and other hydroxylation targets besides HIF-α protein. PHD1 has been shown to regulate the stability of IKKβ whereas ATF4 protein stability was proposed to be determined by PHD3 (157), (160).

Thus, we propose that there might exists further hydroxylation targets or factors that influence PHD2 and regulate its function in addition to pO_2. To answer this question, we initiated a yeast two-hybrid screening using PHD2 as bait. Among the potential interactors we found the FKBP38 protein. FKBP38 is a peptidyl prolyl *cis/trans* isomerase and we speculated that it might function as a PHD2-co-factor to modulate HIF hydroxylation. The goal of this thesis was to investigate the functional role of the FKBP38:PHD2 interaction and its relevance in the PHD-dependent oxygen-sensing pathway.

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5 Publications

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The Peptidyl Prolyl *cis*/*trans* Isomerase FKBP38 Determines Hypoxia-Inducible Transcription Factor Prolyl-4-Hydroxylase PHD2 Protein Stability▽

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The heterodimeric hypoxia-inducible transcription factors (HIFs) are central regulators of the response to low oxygenation. HIF-α subunits are constitutively expressed but rapidly degraded under normoxic conditions. Oxygen-dependent hydroxylation of two conserved prolyl residues by prolyl-4-hydroxylase domain-containing enzymes (PHDs) targets HIF-α for proteasomal destruction. We identified the peptidyl prolyl *cis*/*trans* isomerase FK506-binding protein 38 (FKBP38) as a novel interactor of PHD2. Yeast two-hybrid, glutathione *S*-transferase pull-down, coimmunoprecipitation, colocalization, and mammalian two-hybrid studies confirmed specific FKBP38 interaction with PHD2, but not with PHD1 or PHD3. PHD2 and FKBP38 associated with their N-terminal regions, which contain no known interaction motifs. Neither FKBP38 mRNA nor protein levels were regulated under hypoxic conditions or after PHD inhibition, suggesting that FKBP38 is not a HIF/PHD target. Stable RNA interference-mediated depletion of FKBP38 resulted in increased PHD hydroxylation activity and decreased HIF protein levels and transcriptional activity. Reconstitution of FKBP38 expression abolished these effects, which were independent of the peptidyl prolyl *cis*/*trans* isomerase activity. Downregulation of FKBP38 did not affect PHD2 mRNA levels but prolonged PHD2 protein stability, suggesting that FKBP38 is involved in PHD2 protein regulation.

The response to reduced tissue oxygenation (hypoxia) is characterized by alterations in gene expression, allowing the organism to adapt to hypoxia on the systemic, local, and cellular levels (46). A large number of these target genes are regulated by hypoxia-inducible factor 1 (HIF-1) and HIF-2, the master regulators of oxygen homeostasis in physiological, as well as in pathophysiological, processes (3, 34, 37). HIFs are members of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) transcription factor family and bind as an α/β heterodimer to *cis*-regulatory hypoxia response elements (HREs) (47). Oxygen-dependent regulation is mediated by the HIF-α subunits and involves novel enzymatic posttranslational modification pathways that hydroxylate specific amino acids, depending on the oxygen partial pressure (36). A novel family of oxygen-, ferrous iron-, and 2-oxoglutarate-dependent prolyl-4-hydroxylase domain-containing enzymes (PHD1, PHD2, and PHD3) mediate the proteolytic regulation of HIF-α subunits (4, 9, 15). Under normoxic conditions, two conserved prolyl residues within the oxygen-dependent degradation (ODD) domain of HIF-α are hydroxylated and recognized by the von Hippel-Lindau (pVHL) tumor suppressor protein via *trans*-4-hydroxyprolyl binding (16, 17, 25). pVHL serves as a substrate recognition unit of an E3 ligase complex, targeting HIF-α for degradation by the ubiquitin-proteasome pathway. Because PHDs depend on molecular oxygen as a cosubstrate, hydroxylation is reduced in hypoxia, leading to HIF-α stabilization and accumulation. Thus, PHDs can serve as cellular oxygen sensors and provide a direct link between oxygen availability and HIF-dependent transcriptional regulation. In addition to the regulation of protein stability, hydroxylation of a specific asparaginyl residue within the C-terminal transactivation domain of HIF-α by factor inhibiting HIF (FIH) prevents interaction with the CH1 domains of the p300/CBP transcriptional coactivators, thus reducing the transcriptional activity of HIF-α (13, 21, 22). FIH belongs to the same family as the PHDs, but its K_m for oxygen is lower (14, 20), providing an elegant way of fine tuning HIF-α stabilization and target gene induction by the current tissue oxygenation.

All three PHD proteins are able to hydroxylate HIF-α in vitro, but they differ in their expression patterns, as well as in their subcellular localization, at least when overexpressed (1, 26). However, recent analysis of endogenous localization showed expression of all three PHD enzymes predominantly in the cytoplasm (39). PHD2 is ubiquitously expressed and has been shown to be the main hydroxylase responsible for HIF-α modification in normoxia (2). The recent description of a family with erythrocytosis caused by an inherited PHD2 proline point mutation confirmed these data (32). Interestingly, PHD2 and PHD3 are HIF-dependently regulated, constituting a negative feedback mechanism, whereas PHD1 is not regulated by hypoxia (27, 33). In accordance with the complex functions of HIF in various hypoxic adaptation processes, the PHD pro-

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teins also seem to be subjected to multilayer regulatory mechanisms, including modification of enzyme activity by posttranslational modifications and protein associations (15, 24). To screen for novel PHD interactors, we applied yeast two-hybrid methodology using PHD2 as bait and identified the peptidyl prolyl *cis*/*trans* isomerase FK506-binding protein 38 (FKBP38) as prey.

MATERIALS AND METHODS

Plasmids. Unless otherwise indicated, cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland). Entry vectors were generated by cloning PCR fragments into BamHI/EcoRV-digested (all restriction enzymes were purchased from MBI Fermentas, Labforce, Nunningen, Switzerland) pENTR4 or pENTR/D-TOPO vectors. Full-length PHD1 to -3 (accession numbers NM_053046, NM_022051, and NM_022073, respectively) were amplified by PCR from plasmids pcDNA3.1/PHD1 (29), pcDNA3.1/HA-PHD2 (15), and pcDNA3.1/PHD3 (29). pENTR/PHD2-170-426 was obtained by digesting pENTR/PHD2 with SmaI and NotI, Klenow fill-in, and religation. To obtain pDONR/FKBP38, as well as pDONR/FKBP38Δ98-257, the BP Clonase recombination enzyme mixture (Invitrogen) was used to transfer the corresponding inserts from yeast two-hybrid library vectors into pDONR221. DNA fragments corresponding to amino acids (aa) 3 to 97, aa 99 to 412, and aa 256 to 412 of FKBP38 (accession number AY278607) were amplified by PCR from plasmid pDONR-FKBP38 and cloned into pENTR4. The DNA fragment corresponding to the HIF-2αODD-domain (aa 404 to 569) was amplified by PCR from plasmid pcDNA3/hEPAS and cloned into pENTR/D-TOPO. The inserts of all entry and donor vectors were verified by DNA sequencing (Microsynth, Balgach, Switzerland).

To generate fusion protein expression vectors, entry or donor vectors were recombined in vitro with destination vectors using LR Clonase recombination enzyme mix (Invitrogen). To generate expression plasmids for yeast two-hybrid analysis, the destination vectors pDEST32 (Gal4 DNA-binding domain [Gal4-DBD]) and pDEST22 (Gal4 activation domain [Gal4-AD]) were used. The mammalian Matchmaker vectors pM and pVP16 (Clontech, BD Biosciences, Heidelberg, Germany) were converted to destination vectors by ligation of the Gateway vector conversion cassette reading frame B (Invitrogen) into the EcoRI sites (blunted with Klenow polymerase) of pM and pVP16 to generate pMDEST and pVP16DEST, respectively. Expression vectors for the Gal4-DBD (pM) and VP16AD fusion proteins were obtained after in vitro recombination with the corresponding entry vectors. The mammalian one-hybrid plasmid pM-HIF-1α-370-429-VP16AD was generated by cloning a PCR fragment (using primers 5′-GTCAGAATTCAGAAAATGACTCAGCTATTCACCAA-3′ and 5′-CGATGAATTCGGAATGGTACTTCCTCAAGTTGCT-3′) into the EcoRI site of pM-VP16AD. Plasmids pDEST15 and pDEST17 were used to generate vectors for glutathione *S*-transferase (GST) and His_6 fusion protein expression in bacteria or in rabbit reticulocyte lysates. The plasmid used to generate recombinant GST–HIF-1α-530-826 was described previously (6). pDEST20 was used to generate expression vectors for GST fusion proteins in the baculovirus/Sf9 insect cell system (Invitrogen). pcDNA3.1/nV5-DEST and pcDNA3.1/c-myc-DEST were used to express N-terminal V5- and c-myc-tagged proteins in mammalian cells, respectively. pcDNA3.1/c-myc-DEST was obtained by ligating c-myc oligonucleotides (synthesized by Microsynth) into pcDNA3.1/nV5-DEST digested with EcoRV and HindIII.

Yeast two-hybrid analysis. Yeast two-hybrid analyses were performed using the ProQuest system according to the manufacturer's instructions (Invitrogen). Full-length PHD2 fused to the Gal4-DBD was expressed from pDEST32-PHD2 in *Saccharomyces cerevisiae* strain MaV203 (Invitrogen). To test for self-activity, cotransformants of pDEST32/PHD2 and pExpAD502, encoding the Gal4-AD, were examined on selection plates. A *Gateway*-compatible ProQuest human brain cDNA library was screened on synthetic dropout medium containing 10 mM 3-amino-1,2,4-triazole (Sigma, Buchs, Switzerland). The pExpAD502 plasmids encoding putative PHD2-interacting proteins were isolated, retested for interaction, and tested for self-activity. Inserts of confirmed interactors were sequenced to ensure in-frame coding sequence with the Gal4-AD.

Cell culture and transient transfection. Human Hep3B hepatoma, HeLa cervical carcinoma, and HEK293 embryonic kidney carcinoma cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma) as described previously (6). Transient transfections were performed with the polyethylenimine (Polysciences, Warrington, PA) method (42).

In vitro transcription/translation (IVTT) and GST pull-down. IVTT reactions were carried out as described by the manufacturer (Promega, Madison, WI) using recombined DEST vectors in the presence of [^{35}S]Met (Hartmann Analytic, Braunschweig, Germany). GST and GST fusion proteins were expressed in *Escherichia coli* BL21-AI by induction with 0.025% arabinose for 4 h and affinity purified using glutathione-Sepharose columns (GSTrap FF; GE Healthcare, Dübendorf, Switzerland) by liquid chromatography (BioLogic DuoFlow; Bio-Rad, Reinach, Switzerland). Purified GST-tagged proteins (10 μg) were diluted in bead binding buffer (50 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1% TX-100) and incubated with glutathione-Sepharose beads. For pull-down experiments, 20 μl rabbit reticulocyte IVTT reaction mixture was incubated for 2 h at 4°C with bound GST fusion proteins in coimmunoprecipitation (co-IP) buffer (50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 100 mM NaCl, 0.1% TX-100), washed five times with co-IP buffer, boiled in sample buffer (40 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate [SDS], 50 mM β-mercaptoethanol) for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were stained with Coomassie blue and dried, and radioactively labeled proteins were detected by phosphorimaging (Molecular Imager FX; Bio-Rad).

Immunoblotting. Combined cytoplasmic and nuclear extracts of cultured cells were prepared using 0.4 M NaCl and 0.1% NP-40 in extraction buffer as described previously (23). Nuclear extracts were prepared from isolated nuclei using 0.4 M NaCl. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. Immunoblot analyses were performed as previously described (23). The following antibodies were used: mouse monoclonal antibody (MAb) anti-V5 (Invitrogen), MAb anti-c-myc (Roche Diagnostics, Rotkreuz, Switzerland), MAb anti-β-actin (Sigma), MAb anti-HIF-1α (Transduction Laboratories, BD Biosciences), rabbit polyclonal anti-PHD2 antibody (Novus, Abcam, Cambridge, United Kingdom), rabbit polyclonal anti-FKBP38 antibody (7), and secondary polyclonal goat anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase (Pierce, Perbio, Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce), and signals were recorded with a charge-coupled device camera (FluorChem8900; AlphaInnotech, Witec, Littau, Switzerland) or by exposure to X-ray film (Fujifilm, Dielsdorf, Switzerland).

Co-IP. HEK293 cells were transiently cotransfected with pcDNA3.1/V5-FKBP38 and pcDNA3.1/c-myc-PHD2 using the polyethylenimine method. After 24 h, 10^7 cells were lysed with 1 ml nondenaturing lysis buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% TX-100, complete protease inhibitors [Roche]). The lysates (500 μl) were precleared with protein A agarose beads (Roche) before incubation with protein A agarose beads bound to monoclonal mouse anti-V5 (2 μg; Invitrogen), anti-c-myc (2 μg; Roche), or nonspecific purified mouse immunoglobulin G (IgG) (2 μg; Sigma) overnight at 4°C. Antibody-protein complexes were washed four times with wash buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1% TX-100) and once with phosphate-buffered saline (PBS) and eluted with SDS-PAGE sample buffer.

Immunofluorescence microscopy. Cells were cultivated on microscope slides, washed twice with ice-cold PBS, and fixed on ice for 30 min with 4% paraformaldehyde. The cells were then permeabilized with 0.1% saponin in PBS. Endogenous FKBP38 was detected with rabbit anti-FKBP38 antibodies, and transfected hemagglutinin (HA)-PHD2 was detected with mouse anti-HA antibodies (Sigma). Immune complexes were visualized with goat anti-rabbit antibody–Alexa 488 or goat anti-mouse antibody–Alexa 568 (Molecular Probes, Invitrogen), respectively. Finally, nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) (Sigma) for 30 min. After extensive washings with PBS, the slides were mounted and analyzed by confocal laser scanning microscopy (SP1; Leica Microsystems, Switzerland).

Reporter gene and mammalian one- and two-hybrid assays. Cloning of the HIF-dependent firefly luciferase reporter gene construct pH3SVL was described previously (45). Cells were cotransfected with 500 ng pH3SVL and 20 ng pRL-SV40 *Renilla* luciferase reporter vector (Promega) to control for differences in transfection efficiency and exposed to normoxic or hypoxic conditions for 16 h. Mammalian one- and two-hybrid analyses were performed using the mammalian Matchmaker system (Clontech, BD Biosciences). HeLa cells were transiently cotransfected with one-hybrid or DBD and AD fusion protein vectors, together with the firefly luciferase reporter vector pGRE5xE1b and pRL-SV40. Luciferase reporter gene activity was determined using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega).

RNAi. For silencing FKBP38 by RNA interference (RNAi), a pair of 63-nucleotide oligonucleotides (Microsynth) targeting the sequence 5′-AAGAGUGGCUGGACAUUCUGG-3′ were inserted into pSilencer2.1-U6 hygro digested with BamHI and HindIII (Ambion, Huntingdon, United Kingdom) and transfected into HeLa and Hep3B cells by calcium phosphate coprecipitation. A

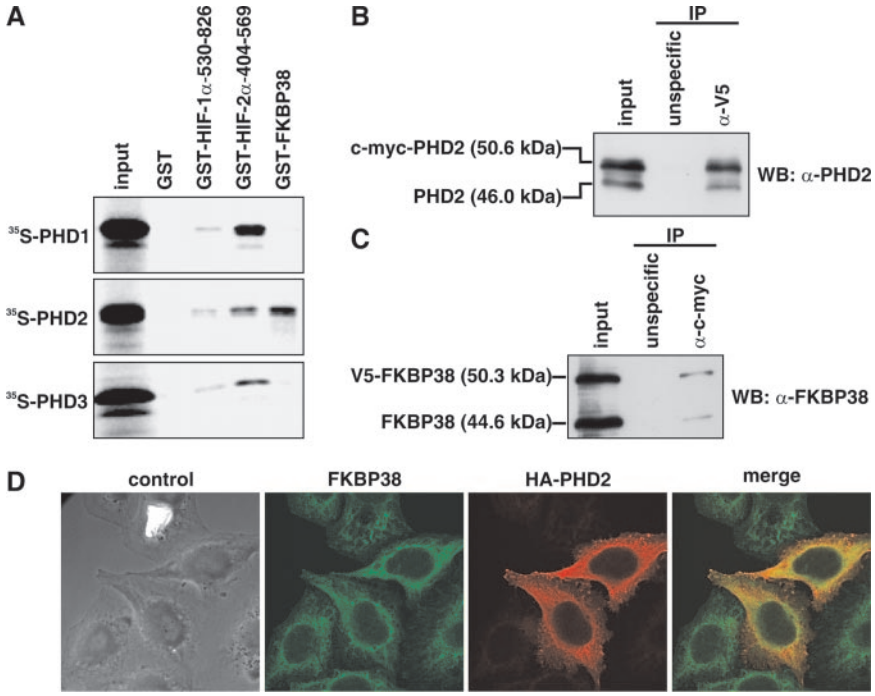

FIG. 1. FKBP38 interacts specifically with PHD2. (A) IVTT ^{35}S-labeled PHD1, PHD2, and PHD3 were incubated with GST–HIF-1α-530-826, GST–HIF-2α-404-569, GST-FKBP38, or GST alone. Bound proteins were pulled down and visualized by phosphorimaging. (B and C) Total cell extracts from HEK293 cells transiently transfected with V5-FKBP38 and c-myc–PHD2 were incubated with anti-V5 (B) or anti-c-myc (C) antibodies or with isotype-matched control IgG. The antibodies and bound proteins were immunoprecipitated and analyzed by immunoblotting (WB). Coimmunoprecipitated PHD2 and FKBP38 were detected by specific antibodies. IP, immunoprecipitate. (D) Indirect immunofluorescence of FKBP38 and PHD2. HeLa cells were transfected with a plasmid encoding HA-PHD2 and stained with antibodies to FKBP38 (green) and HA (red), followed by fluorescently labeled secondary antibodies.

pool of clones were selected in medium containing 200 μg/ml hygromycin B (Calbiochem, VWR International, Lucerne, Switzerland), and single clones were subsequently isolated by limited dilution. The downregulation of FKBP38 expression by RNAi was analyzed by real-time reverse transcriptase (RT) PCR and immunoblotting and compared to cells containing the control oligonucleotide harboring the vector pSilencer2.1-U6 hygro.

Pulse-chase experiment. HeLa cells were starved in DMEM (without fetal calf serum [FCS]) lacking Met and Cys for 1 h. The cells were then incubated in the same medium supplemented with 10% FCS (dialyzed against PBS) and [^{35}S]Met/Cys mixture (0.1 mCi/ml; Hartman Analytics, Braunschweig, Germany) for 2 h. After the cells were washed twice with PBS, they were chased with DMEM-10% FCS containing cold Met/Cys (3 mM each). During the chase period, cells were harvested at different time points, and radioactively labeled PHD2 was immunoprecipitated and visualized by phosphorimaging.

In vitro prolyl-4-hydroxylation assay. HeLa cells were pelleted and Dounce homogenized in cell lysis buffer (100 mM Tris-Cl, pH 7.5, 1.5 mM $MgCl_2$, 8.75% glycerol, 0.01% Tween 20) supplemented with complete protease inhibitors. The lysates were centrifuged for 30 min at 4°C and 20,000 × *g*, and pVHL-elongin B-elongin C (VBC) binding was determined as previously described (23, 30).

RNA extraction and real-time RT-PCR quantification. Cells were grown in 15-cm plates under normoxic or hypoxic conditions, and total RNA was extracted as described previously (23). First-strand cDNA synthesis was performed with 3 μg of RNA using Superscript III Moloney murine leukemia virus RT according to the manufacturer's instructions (Invitrogen). Subsequently, mRNA expression levels were quantified with 2 μl of diluted cDNA reaction mixture by real-time PCR using a SybrGreen Q-PCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, ribosomal protein L28 mRNA was determined, and the data were expressed as ratios relative to L28 levels. Primers were as follows: hL28 forward, 5′-GCAATTCCTTCCGCTACAAC-3′; hL28 reverse, 5′-TGTTCTTGCGGATCATGTGT-3′; hGLUT1 forward, 5′-TCACTGTGCTCCTGGTTCTG-3′; hGLUT1 reverse, 5′-CCTGTGCTCCTGAGAGATCC-3′; hCAIX forward, 5′-GGGTGTCATCTGGACTGTGTT-3′; hCAIX reverse, 5′-CTTCTGTGCTGCCTTCTCACT-3′; hFKBP38 forward, 5′-ACATGACGTTCGAGGAGGAG-3′; hFKBP38 reverse, 5′-GTTGGAAGGTTCCAGCTTCA-3′; hPHD1 forward, 5′-CTGGGCAGCTATGTCATCAA-3′; hPHD1 reverse, 5′-AAATGAGCAACCGGTCAAAG-3′; hPHD2 forward, 5′-GAAAGCCATGGTTGCTTGTT-3′; hPHD2 reverse, 5′-TTGCCTTCTGGAAAAATTCG-3′; hPHD3 forward, 5′-ATCGACAGGCTGGTCCTCTA-3′; and hPHD3 reverse, 5′-CTTGGCATCCCAATTCTTGT-3′.

RESULTS

Identification of FKBP38 as a specific PHD2 interactor. To identify novel PHD2-interacting proteins, human PHD2 expressed as a fusion protein with the Gal4-DBD was used as bait in a yeast two-hybrid screen of a human brain cDNA library. A total of 2×10^6 individual transformants were screened, and several independent cDNA clones encoding FKBP38 were identified based on their abilities to activate the three reporter genes *His*, *Ura*, and *LacZ* (data not shown). In order to confirm these data in a yeast-independent interaction assay, GST pull-down experiments were performed. PHD1, -2, and -3 were subjected to IVTT in the presence of radioactive [^{35}S]Met, incubated with bacterially expressed and purified GST-FKBP38, and precipitated by glutathione-Sepharose. As controls, GST–HIF-1α-530-826 and GST–HIF-2α-404-569 were also expressed and purified as GST fusion proteins. GST alone was used as a noninteraction control. Whereas HIF-α fragments containing the ODD domain interacted with all three PHDs, FKBP38 showed specific association with PHD2 only (Fig. 1A), confirming the previous findings in yeast (data not

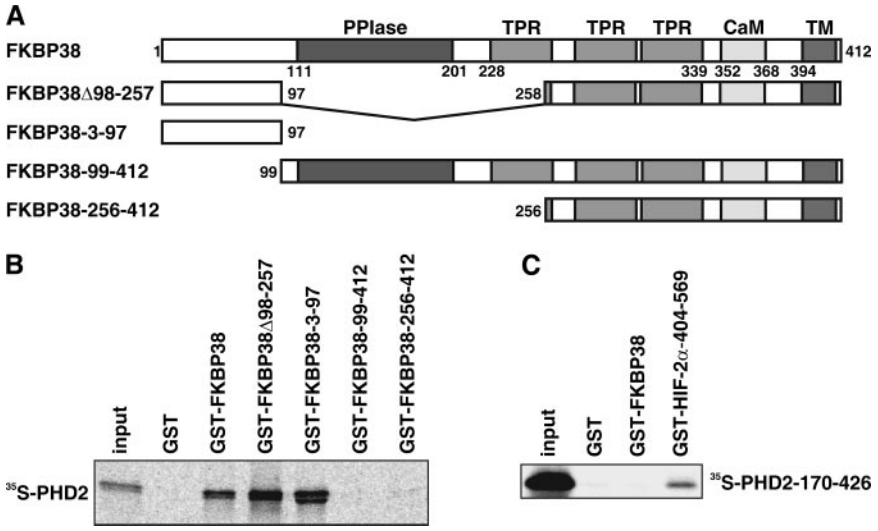

FIG. 2. Mapping of the FKBP38 and PHD2 interaction sites. (A) Schematic representation of the predicted FKBP38 domain architecture and the FKBP38 constructs used. TPR, tetratricopeptide repeats; CaM, calmodulin-binding site; TM, transmembrane domain. (B) IVTT ^{35}S-labeled PHD2 was allowed to interact with GST-FKBP38, GST–FKBP38Δ98-257, GST–FKBP38-3-97, GST–FKBP38-99-412, GST–FKBP38-256-412, or GST alone; the protein complexes were precipitated by glutathione-Sepharose, separated by SDS-PAGE, and visualized by phosphorimaging. (C) IVTT ^{35}S-labeled PHD2-170-426 was tested for interaction with GST-FKBP38, GST–HIF-2α-404-569, or GST alone and analyzed as described above.

shown). The stronger binding of PHD2 to HIF-2α-404-569 than to HIF-1α-530-603 might be due to the presence of both hydroxylated proline residues in the recombinant HIF-2α protein (P405 and P531) versus just the C-terminal proline in HIF-1α (P564).

To confirm the FKBP38:PHD2 interaction in a mammalian system, we transiently transfected HEK293 cells with V5-tagged FKBP38 and c-myc-tagged PHD2. Tag-specific antibodies were then used for co-IPs. V5-FKBP38 interacted with exogenous, as well as with endogenous, PHD2 (Fig. 1B), and c-myc–PHD2 was found in a complex with both overexpressed and cellular FKBP38 (Fig. 1C). Nonspecific isotype-matched mouse IgG served as an immunoprecipitation control.

We next investigated whether FKBP38 colocalizes with PHD2 in HeLa cells. Since the antibodies directed against these two proteins were generated in rabbits, we transiently transfected HA-PHD2 and used mouse anti-HA antibodies to detect PHD2. Immunofluorescence analysis indicated that endogenous FKBP38 colocalized with HA-PHD2 in the cytoplasm of HeLa cells (Fig. 1D).

Mapping of the PHD2 interaction site in FKBP38. FKBP38 belongs to the enzyme class of peptidyl prolyl *cis*/*trans* isomerases (PPIases), and the amino-terminal region of FKBP38 contains a PPIase domain of the FKBP type, harboring PPIase- and FK506-binding activities. In addition, FKBP38 contains three tetratricopeptide repeats, a calmodulin-binding site, and a membrane anchor in its carboxy-terminal half (7, 38). Based on the predicted domain architecture of FKBP38 (Fig. 2A), a series of deletion mutants fused to GST were designed, expressed in bacteria, purified, and analyzed for PHD2 interaction in GST pull-down experiments. Radioactively labeled PHD2 was produced by IVTT, and the interaction of labeled PHD2 with purified GST-FKBP38 deletion constructs, or GST alone, was tested. The N-terminal FKBP38 fragment containing aa 3 to 97 was sufficient to maintain interaction with PHD2 (Fig. 2B). The PPIase domain was not required for PHD2 interaction. On the other hand, an N-terminally truncated PHD2 protein (PHD2 aa 170 to 426) interacted with GST–HIF-2α-404-569 but not with GST-FKBP38 (Fig. 2C). Similar results were obtained in yeast two-hybrid experiments (data not shown). These data suggest that aa 3 to 97 of FKBP38 are required for interaction with aa 1 to 169 of PHD2. The catalytic domain of purified PHD2 is sufficient for HIF-2α ODD domain binding and hydroxylation of HIF-1α-derived peptides (data not shown), suggesting that FKBP38 is not a PHD2 hydroxylation substrate.

FKBP38-PHD2 interaction and FKBP38 gene expression are independent of the oxygen concentration. To investigate whether the FKBP38-PHD2 interaction is oxygen-dependent, we expressed PHD1, PHD2, or PHD3 fused to the Gal4-DBD, together with the VP16AD fused to FKBP38, in a mammalian two-hybrid system in HeLa cells. The activity of a cotransfected luciferase reporter gene construct containing five Gal4-DBD sites is greatly enhanced when the AD and DBD fusion proteins interact with each other. As shown in Fig. 3A, luciferase expression was significantly higher when the DBD-PHD2 and AD-FKBP38 fusion constructs were cotransfected than in transfection of either construct alone or AD-FKBP38 in combination with DBD-PHD1 or DBD-PHD3, confirming specific FKBP38-PHD2 interaction in mammalian cells. Cotransfection of DBD-PHD2 with the AD construct fused to FKBP38 deletions confirmed the findings in yeast, as well as the in vitro interaction results mentioned above (data not shown). DBD-p53, together with AD-CP (polyomavirus coat protein), served as a negative control. In contrast to the significantly increased luciferase activity under hypoxic conditions following cotransfection of DBD-PHD2 with AD–HIF-2α-404-569 (HIF-2αODD), luciferase expression remained unchanged following cotransfection of DBD-PHD2 with AD-FKBP38 under hypoxic and normoxic conditions.

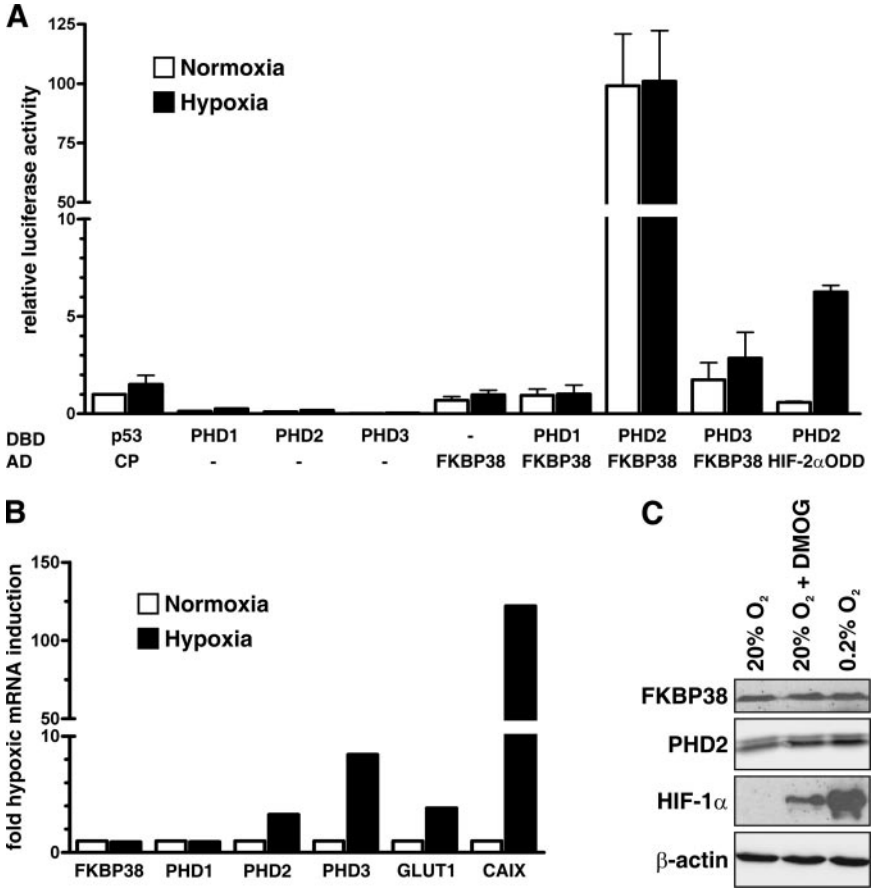

FIG. 3. FKBP38-PHD2 interaction and FKBP38 gene expression are not regulated by oxygen. (A) HeLa cells were transiently transfected with Gal4-DBD and VP16AD fusion protein vectors and Gal4 response element-driven firefly luciferase reporter, as well as a *Renilla* luciferase control vector. Following transfection, the cells were incubated under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions, and luciferase reporter gene activities were determined 16 h later. Firefly/*Renilla* luciferase activity ratios were normalized to the normoxic negative control DBD-p53/AD-CP cotransfection, which was arbitrarily defined as 1. Mean values plus standard errors of the mean are shown for three independent experiments performed in triplicate. (B and C) HeLa cells were cultured under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions or treated with the PHD inhibitor dimethyloxalylglycine (DMOG) (2 mM). (B) Total RNA was extracted after 8 h of incubation, and mRNA levels of FKBP38, PHD1 to -3, GLUT1, CAIX, and ribosomal protein L28 were quantified by real-time RT-PCR. The transcript levels of these genes were normalized to L28, and hypoxic inductions were calculated (mean, $n = 2$). (C) Cellular proteins were extracted and separated by SDS-PAGE, and endogenous FKBP38, PHD2, HIF-1α, and β-actin levels were analyzed by immunoblotting.

We next determined mRNA levels in cultured HeLa cells exposed to normoxic or hypoxic conditions by real-time RT-PCR. The known oxygen-regulated HIF target genes PHD2, PHD3, GLUT1, and CAIX, but not FKBP38, PHD1, or L28 control mRNA, were induced by hypoxia (Fig. 3B). FKBP38 protein levels were not regulated by inhibition of PHDs with dimethyloxalylglycine or by hypoxia, whereas HIF-1α, as well as PHD2, protein expression was increased following PHD inhibition or hypoxic exposure (Fig. 3C). In summary, these data demonstrate an oxygen-independent interaction of FKBP38 with PHD2, suggesting that FKBP38 is not a PHD2 substrate but might rather act as a cofactor.

RNAi-mediated FKBP38 silencing increases PHD-dependent HIF-1α hydroxylation. To investigate the functional consequences of the FKBP38-PHD2 interaction with regard to the regulation of PHD activity, FKBP38 mRNA was downregulated by RNAi. HeLa and Hep3B cells were transfected with a FKBP38-silencing construct or with a control plasmid harboring nonspecific oligonucleotides. Pools of clones were generated by hygromycin B selection, and single clones were isolated by limited dilution. As shown in Fig. 4A and B, respectively, FKBP38 RNAi expression resulted in decreased endogenous FKBP38 mRNA, as well as protein levels, in HeLa cells. Similar data were obtained in Hep3B cells (data not shown). The ability of cell lysates derived from FKBP38 RNAi clones to hydroxylate HIF-1α was investigated in an in vitro prolyl-4-hydroxylation assay. PHD hydroxylation activity was measured by binding of the VBC complex to HIF-1α-derived peptides. Decreased FKBP38 resulted in a concomitant significant increase in hydroxylation activity (Fig. 4C). Reconstitution of FKBP38 gene expression using transiently transfected expression vectors normalized prolyl hydroxylation (Fig. 4D), whereas transfection with a *lacZ* expression vector had no effect (data not shown).

Expression of a functional mutant FKBP38ΔPPIase (FKBP 38Δ98-257) resulted in normalized prolyl hydroxylation comparable to transfection with FKBP38 (data not shown).

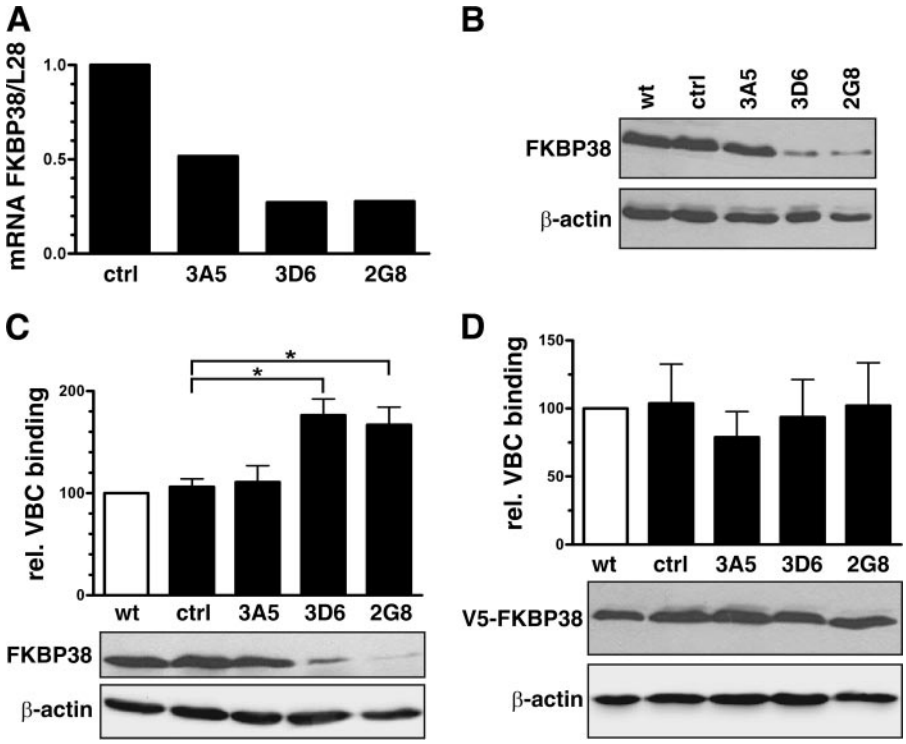

FIG. 4. Enhanced hydroxylation activity by RNAi-mediated FKBP38 depletion. HeLa cells were transfected with vectors containing FKBP38 RNAi or control oligonucleotides, and stable clones were selected (3A5, 3D6, and 2G8). (A) Total RNA was extracted, and FKBP38 transcript levels were determined by real-time RT-PCR and normalized to L28 mRNA levels (mean, $n = 2$). (B) Total cell extracts were prepared and analyzed by immunoblotting for FKBP38 and β-actin expression. Total cell extracts of untransfected (C) and FKBP38-reconstituted (D) stable RNAi clones were prepared, and hydroxylation activity was measured using the VBC-binding assay. Shown are mean values of relative VBC binding plus standard errors of the mean of three independent experiments performed in triplicate. *P* values were obtained by paired *t* tests (*, $P < 0.05$). Subsequently, cell extracts were analyzed by immunoblotting for endogenous FKBP38, transfected V5-FKBP38, and β-actin expression. wt, wild type; ctrl, control.

FKBP38 silencing reduces HIF-1α-dependent reporter gene activity. To further investigate whether increased hydroxylation activity due to FKBP38 depletion affects HIF-dependent reporter gene expression, wild-type HeLa, as well as FKBP38, small interfering RNA (siRNA) cell clones were transiently cotransfected with pH3SVL, containing six HIF-binding sites from the transferrin HRE and a *Renilla* luciferase control plasmid. Luciferase activity was reduced under hypoxic conditions in FKBP38-depleted cell clones compared with control cells (Fig. 5A). It has recently been reported that PHD2 is able to modulate HIF-1α transcriptional activity (44), and therefore, we established a mammalian one-hybrid assay to investigate HIF-1α protein stability directly. FKBP38 siRNA cell clones were transiently cotransfected with HIF-1α-370-429 fused at the N-terminal end with Gal4-DBD and at the C-terminal end with VP16AD, a firefly luciferase reporter gene construct containing five Gal4-DBDs, and a *Renilla* luciferase control plasmid. As shown in Fig. 5B, luciferase activity was significantly reduced under normoxic as well as hypoxic conditions in FKBP38-depleted cell clones compared with control cells. Reconstitution of V5-FKBP38 with a FKBP38 expression vector, but not with a *lacZ* control vector, rescued luciferase activity (Fig. 5C). Overexpression of a functional FKBP38 mutant that does not bind PHD2 (FKBP38-99-412) resulted in luciferase activities comparable to the *lacZ* control transfections (Fig. 5C). The nonimmunosuppressive small molecule GPI1046 [3-(3-pyridyl)-1-propyl (2*S*)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidine carboxylate] mimics the FK506 residues interacting with FKBP38. GPI1046 has been shown to preferentially bind FKBP38 and to inhibit the PPIase activity with a K_i of 48 nM (7, 40). Addition of GPI1046, to inhibit FKBP38 PPIase activity, to V5-FKBP38 reconstituted cells had no effect, indicating a PPIase-independent function of FKBP38 in the regulation of HIF-1α hydroxylation (data not shown). Note that relative luciferase activities were generally higher in functional FKBP38 reconstituted clones than in cells transfected with FKBP38, mutant for PHD2 binding, or *lacZ*, suggesting that overexpression of FKBP38 reduces PHD-dependent degradation of the HIF one-hybrid construct.

Reduced FKBP38 gene expression leads to increased PHD2 protein levels and attenuates HIF-dependent gene expression. The enhanced hydroxylation and reduced HIF-1α-dependent one-hybrid reporter gene activity found in FKBP38-depleted cells could be based on modification of the enzymatic activity itself and/or on elevated enzyme abundance. However, incubation of active GST-PHD2 fusion protein purified from baculovirus-infected Sf9 cells with recombinant GST-FKBP38 did not influence PHD2 hydroxylation activity as measured by the

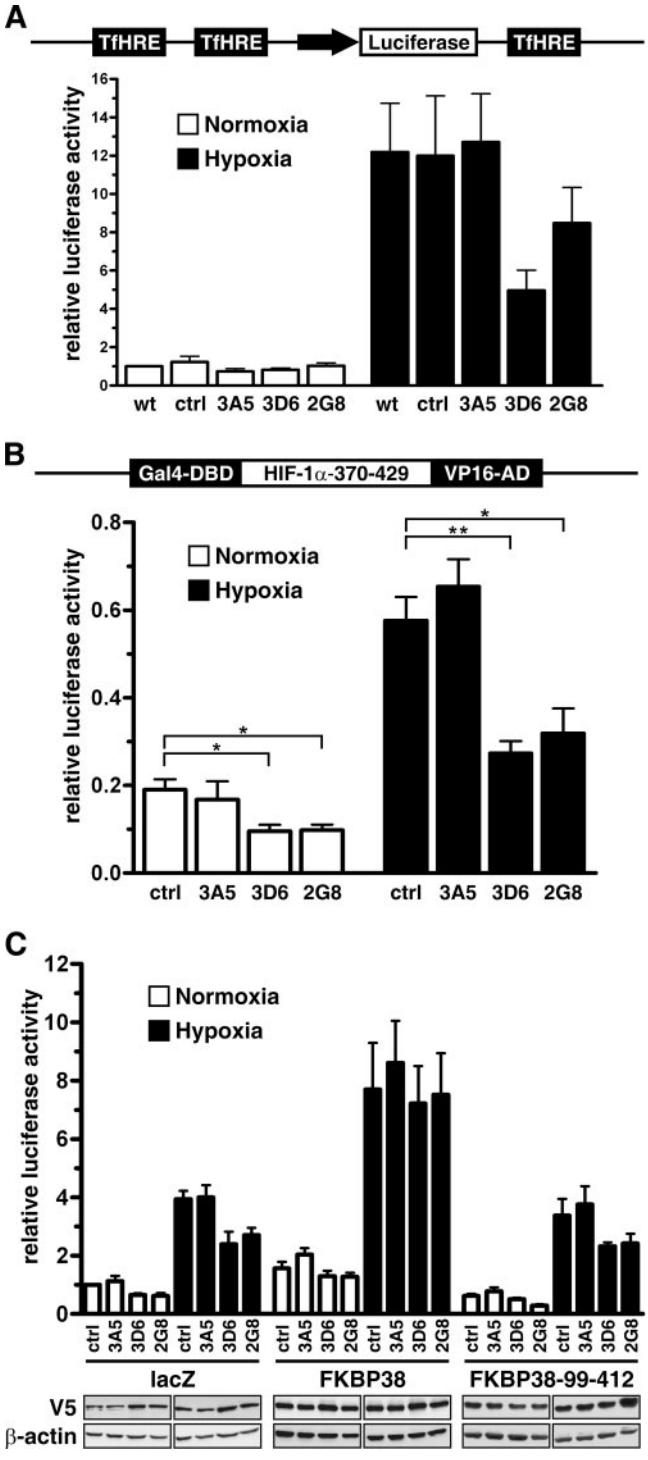

FIG. 5. FKBP38 regulates HIF-dependent reporter gene activity. (A) HeLa wild-type (wt) as well as FKBP38 siRNA cell clones were cotransfected with pH3SVL and pRL-SV40 *Renilla* luciferase reporter vectors and cultivated for 16 h under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions before relative luciferase activities were determined. The results are mean values plus standard errors of the mean of five independent experiments performed in triplicate. ctrl, control. (B) Stable FKBP38 RNAi HeLa clones were transiently transfected with Gal4-DBD–HIF-1α-370-429–VP16AD expression vectors (schematically represented) and Gal4 response element-driven firefly luciferase reporter, as well as a *Renilla* luciferase control vector. (C) HeLa clones were cotransfected with the one-hybrid reporter gene vectors and FKBP38, FKBP38-99-412, or *lacZ* control expression vectors. Eight hours posttransfection, the cells were cultured under either normoxic or hypoxic conditions for an additional 16 h, and firefly luciferase activities were determined and corrected for *Renilla* luciferase activity. The results are mean values of relative luciferase activities plus standard errors of the mean of at least three independent experiments performed in triplicate. *P* values were obtained by paired *t* tests (**, $P < 0.01$; *, $P < 0.05$). Expression of the transfected V5-tagged vectors was verified by immunoblotting against V5 and β-actin.

VBC capture assay (data not shown). Therefore, we analyzed PHD2 protein levels in FKBP38 RNAi cell clones and observed enhanced PHD2 levels inversely related to FKBP38 (Fig. 6A, left). Transient transfection of these FKBP38 RNAi cells with V5-FKBP38 restored the low constitutive PHD2 protein levels, comparable to those of wild-type HeLa cells (Fig. 6A, middle). Addition of GPI1046 did not influence PHD2 protein levels in wild-type HeLa or FKBP38 RNAi cell clones, again indicating a PPIase-independent effect (Fig. 6A, right). The same results were obtained by treating cells with *N*-(*N*′,*N*′-dimethylcarboxamidomethyl)-cycloheximide, another FKBP38 PPIase inhibitor (reference 8 and data not shown).

PHD1, PHD2, and PHD3 mRNA levels were not affected by downregulation of FKBP38, indicating that PHD2 is posttranscriptionally regulated by FKBP38 (Fig. 6B). To analyze whether FKBP38 is able to attenuate endogenous HIF-1α protein expression by modification of PHD2 abundance, HeLa control cells and FKBP38 RNAi cell clones were cultured under hypoxic conditions and analyzed by immunoblotting. PHD2 protein levels were inversely related to FKBP38 expression even under hypoxic conditions (Fig. 6C). Note that elevated PHD2 protein levels in FKBP38-silenced cells compared to control cells are less pronounced, because hypoxia upregulated PHD2 expression in the control cells. HIF-1α protein was not detectable under normoxic conditions but strongly increased in hypoxic control cells. Strikingly, HIF-1α protein levels markedly decreased in hypoxic FKBP38-depleted cells, suggesting that elevated PHD2 levels lead to increased proteolytic destruction of HIF-1α, even under hypoxic conditions (Fig. 6C).

Reduced HIF-1α protein levels might explain the less pronounced increase in PHD2 levels under hypoxic conditions. Indeed, hypoxia-inducible transcripts of HIF target genes, including PHD2, PHD3, and GLUT-1 but not PHD1, were attenuated two- to threefold in FKBP38 knockdown cell clones (Fig. 6D). These results indicate that increased PHD2 abundance in FKBP38 RNAi cell clones led to elevated HIF-α substrate hydroxylation and subsequent degradation, finally resulting in decreased HIF function in the cell.

PHD2 protein stability is increased in FKBP38-downregulated cells. Increased PHD2 protein levels in FKBP38-depleted cells could be due to increased translation or protein stability. To analyze PHD2 protein stability, we performed pulse-chase experiments in wild-type HeLa and FKBP38-silenced HeLa 2G8 cells (Fig. 7A). Whereas the measured half-life of PHD2 was about 20 h in wild-type cells, it was extrapolated to approximately twice as long when FKBP38 was downregulated. In addition, cycloheximide treatment and subsequent analysis of PHD2 protein levels showed a half-

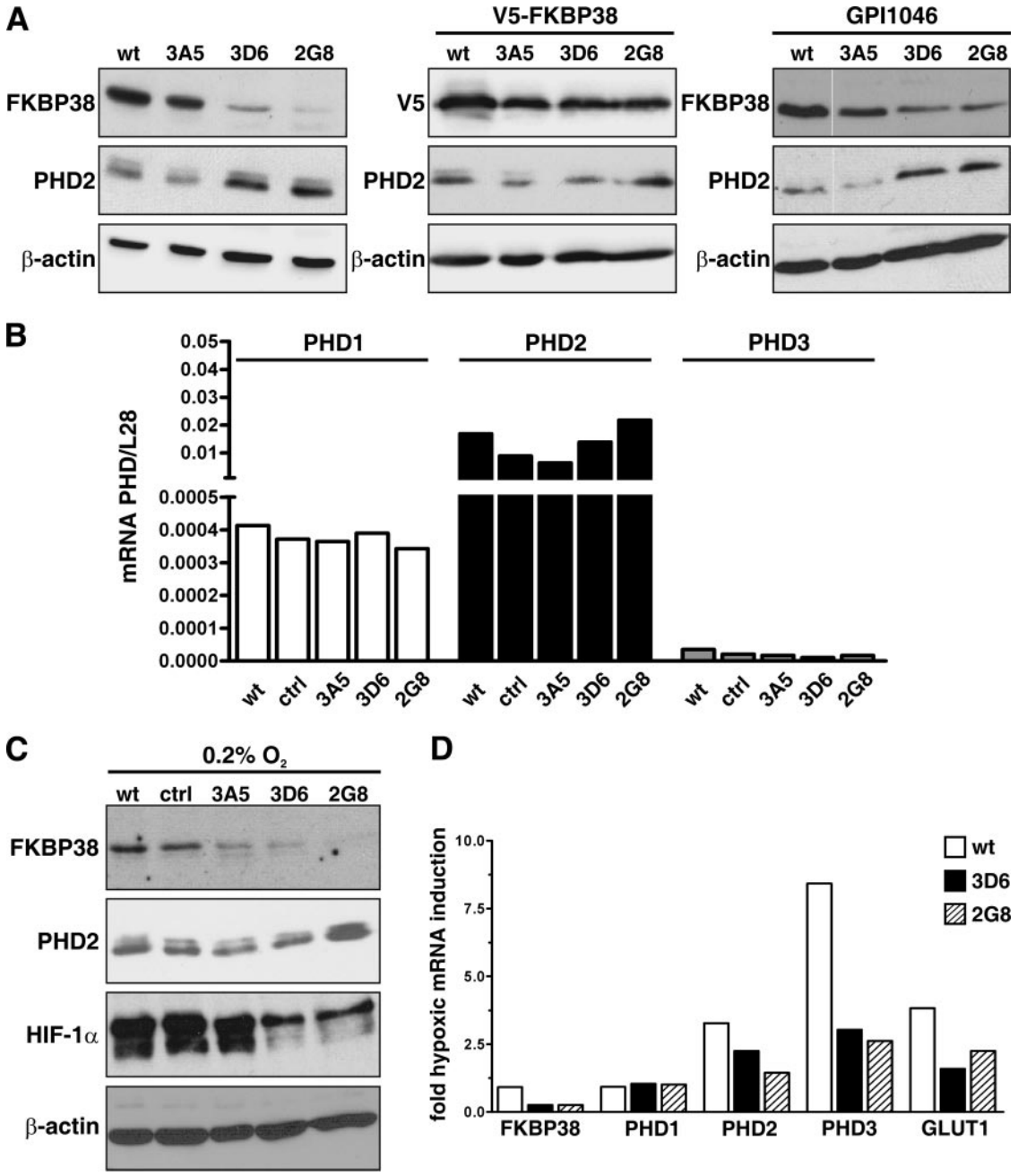

FIG. 6. Increased PHD2 protein stability in FKBP38-depleted cells. (A) Wild-type HeLa cells (wt), stable FKBP38-silenced HeLa clones, V5-FKBP38 reconstituted HeLa cells, or FKBP38-silenced HeLa cells treated with the FKBP38 inhibitor GPI1046 (10 μM) were cultivated under normoxic (20% O_2) conditions, and FKBP38, V5-FKBP38, PHD2, or β-actin was detected by immunoblotting. (B) Total RNA was extracted and reverse transcribed into cDNA, and mRNA levels of PHD1, PHD2, and PHD3 were normalized to ribosomal L28 mRNA levels as quantified by real-time RT-PCR ($n = 2$). ctrl, control. (C) Total hypoxic FKBP38 RNAi cell clone lysates were separated by SDS-PAGE and analyzed for FKBP38, PHD2, HIF-1α, and β-actin protein levels by immunoblotting. (D) Total RNA was extracted after 8 h of incubation in normoxia or hypoxia, and mRNA levels of FKBP38, PHD1 to -3, GLUT1, and ribosomal L28 were quantified by real-time RT-PCR. The transcript levels of these genes were normalized to L28 mRNA, and hypoxic inductions were calculated ($n = 2$).

life in the same range, as determined by pulse-chase in control HeLa cells, whereas PHD2 protein stability was markedly elevated in FKBP38-downregulated cell clones (Fig. 7B). These experiments suggest a function of FKBP38 in the proteolytic regulation of PHD2. FKBP38 protein levels remained constant for up to 32 h during cycloheximide treatment (Fig. 7C). PHD2 is induced in HeLa cells by incubation under hypoxic conditions for 24 h. After reoxygenation, PHD2 levels remain increased for up to 24 h and afterwards slowly decline. In contrast, in FKBP38-downregulated HeLa cells, PHD2 remained elevated even after 72 h of reoxygenation (Fig. 7D), indicating that FKBP38 is

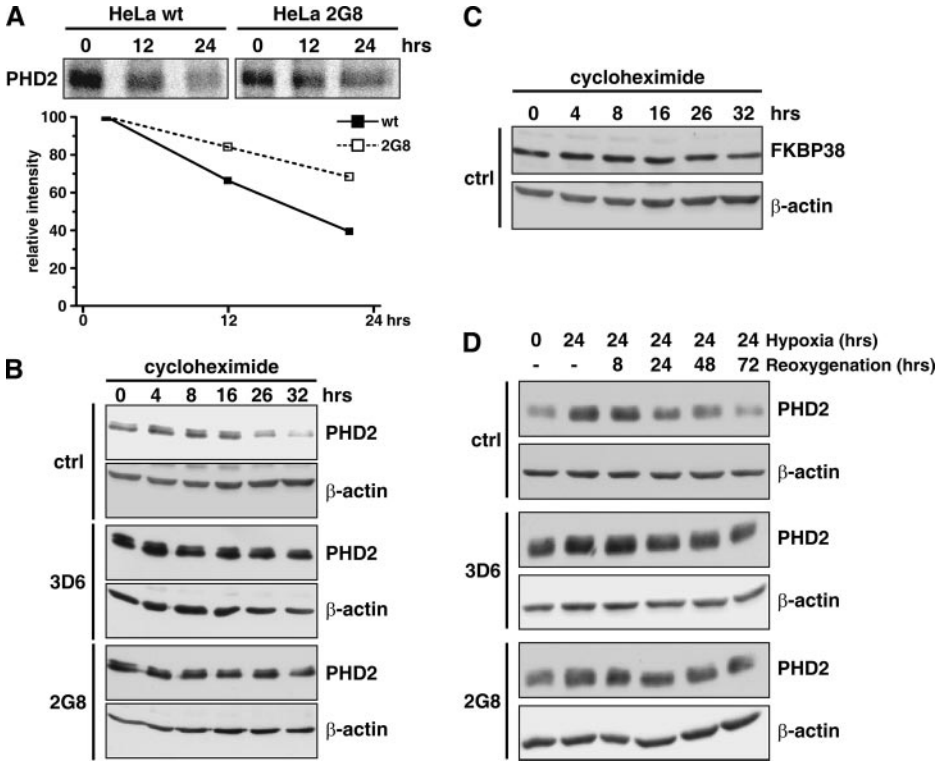

FIG. 7. Increased PHD2 protein stability in FKBP38-silenced cells. (A) The half-lives of PHD2 protein in wild-type HeLa (wt) and FKBP38-depleted (2G8) cells were monitored by a pulse-chase experiment. Cells were metabolically labeled with [^{35}S]Met/Cys for 2 h, followed by chase for the indicated times. PHD2 was immunoprecipitated from the cell lysates with anti-PHD2 antibodies, and signals were detected by autoradiography. Translation was blocked in FKBP38 RNAi HeLa clones by addition of 100 μM cycloheximide and PHD2 (B), as well as FKBP38 (C), and protein levels were determined after the indicated time periods by immunoblotting. Subsequent detection of β-actin served as a loading control. (D) Total lysates of HeLa cells were prepared at the indicated periods of hypoxia or hypoxia followed by reoxygenation, and PHD2 as well as β-actin levels were detected by immunoblotting.

also required for the regulation of PHD2 protein levels under reoxygenation conditions.

DISCUSSION

Although oxygen-dependent hydroxylation directly links oxygen availability to HIF-α protein stabilization and transcriptional activity, both HIF and PHD hydroxylase activities are likely to be subjected to additional levels of regulation. Indeed, a HIF-dependent regulatory feedback mechanism has been shown to increase PHD2 and PHD3 protein abundance under hypoxic conditions, suggesting a role during reoxygenation (9). We recently found that inducible PHD2 and PHD3 mRNA levels remain upregulated under prolonged hypoxic exposure and that PHDs maintain a functional HIF-α hydroxylation activity even under severe hypoxic conditions (42). Biochemical analysis of a PHD2 protein complex, consisting of approximately 15 proteins with an apparent molecular mass of 320 to 440 kDa, indicated that PHD2 might be part of a large heteromeric complex (15). The formation of stable complexes with other proteins raises the possibility that such proteins might be involved in the regulation of PHD2 function, e.g., through chaperones; modulation of access to the cosubstrates oxygen, ferrous iron, 2-oxoglutarate, and probably ascorbate; and target protein recognition. Because HIF prolyl hydroxylation is a nonreversible process, differences in PHD protein abundance would also be a means of regulating hydroxylation activity.

We identified FKBP38 as a PHD2-associated nonsubstrate protein and showed that silencing of FKBP38 results in increased PHD2 stability, leading to enhanced hydroxylation and attenuated HIF-dependent transcription. Conversely, FKBP38 overexpression resulted in elevated HIF reporter gene activity in transient transfections (data not shown), as well as in one-hybrid assays (Fig. 5C). Whereas PHD1 and PHD3 are targeted for proteasomal degradation by the E3 ubiquitin ligases Siah1a and Siah2 under hypoxic conditions, it is so far unknown how PHD2 is posttranslationally regulated (28). Although the molecular mechanism remains elusive, FKBP38-dependent decreased PHD2 protein stability provides the first data concerning the proteolytic regulation of PHD2 and attributes a novel function to FKBP38.

The enzyme class of PPIases encompasses the immunophilin families of cyclophilins and FKBPs, which were originally discovered as cellular receptors of the immunosuppressive drugs cyclosporine A and FK506, respectively (11, 43). Although structurally unrelated, the two subfamilies share the common enzymatic activity to catalyze the isomerization of the *cis* and *trans* conformers of peptide bonds preceding prolines (10). The singularity of peptidyl-prolyl bonds in peptide chains is caused by the formation of an imidic peptide bond at the N

terminus of the N-alkylated amino acid proline that is unique among amino acids. The rate of the uncatalyzed interconversion between the prolyl *cis*/*trans* isomers is low, pointing to an involvement of PPIases in protein folding. It has been shown for collagen that proline hydroxylation occurs on the nascent polypeptide chain during translation, and subsequent cyclophilin-assisted *cis*/*trans* isomerization is the rate-limiting step for collagen helix formation. Procollagen folding is slowed down by the addition of cyclosporine A (41).

Because VHL binds to HIF-α *trans*-4-hydroxyprolyl residues (16, 17), we speculated that the PPIase FKBP38 might act as a PHD2 cofactor, influencing the conformation of HIF-α prolyl residues and/or the activity of PHD2. However, FKBP38 did not physically interact with HIF-α in yeast or mammalian two-hybrid assays (data not shown), suggesting that FKBP38 does not influence the conformation of the HIF-α ODD domain. On the other hand, the PPIase domain of FKBP38 was not required for PHD2 interaction (Fig. 2), Ca^{2+}/calmodulin-mediated activation of recombinant FKBP38 did not modulate the hydroxylation capacity of PHD2 (data not shown), and the small-molecule FKBP38 PPIase inhibitor GPI1046, as well as *N*-(*N*′,*N*′-dimethylcarboxamidomethyl)-cycloheximide, did not influence PHD2 protein abundance (Fig. 6A and data not shown, respectively). These data provide evidence that the effect of FKBP38 on PHD2 abundance is PPIase independent.

FKBP38 is widely expressed in both adult and embryonic tissues, as well as in different cancer cell lines (5, 18). Recently, knockout mice showed that FKBP38 plays an essential role during development. FKBP38 acts as a negative regulator of the sonic hedgehog (SHH) pathway, and its loss leads to ectopic and ligand-independent activation of SHH signaling, resulting in disturbed cell fate determination and embryonic lethality (5). However, the molecular function of FKBP38 in the SHH pathway remains elusive, and it will be interesting to determine whether PHD2 is involved.

An interaction of FKBP38 with Bcl-2 has also been described (38), but the functional consequences of this association remain controversial. Antiapoptotic (38), as well as proapoptotic (7), effects have been reported, probably depending on the cellular context. The two proteins interacted only upon Ca^{2+} influx and subsequent FKBP38 activation. However, we did not observe a cofactor-dependent interaction of FKBP38 with PHD2, nor did the addition of Ca^{2+} to cell lysates alter the hydroxylation activity, and cell viability was not affected by RNAi-mediated downregulation of FKBP38 as measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays (data not shown). Further potential functions of FKBP38 include antitumor effects by regulation of anti-invasive syndecan 1 and suppression of proinvasive MMP9 and regulation of cell size via the tuberous sclerosis complex (12, 35). Clearly, the increasing amount of data about potential FKBP38 functions suggests that FKBP38 is a multifunctional protein involved in the regulation of many cellular processes, but further studies are required for a better comprehension of the biochemical functions of FKBP38.

In addition to FKBP38, the candidate tumor suppressor ING4 was shown to associate with PHD2, suppressing HIF activity and thereby possibly attenuating HIF target genes involved in tumor growth and angiogenesis (31). It has been proposed that ING4 retains PHD2 in the nucleus in order to degrade HIF upon reoxygenation. However, recent data showed predominant cytoplasmic localization of PHD2 (39). PHD2 has a calculated molecular mass of 46 kDa, enabling it to pass through nuclear pores, and it might be actively excluded from the nucleus by interaction with FKBP38. FKBP38 contains a C-terminal transmembrane domain that anchors the protein in the mitochondrial and endoplasmic reticulum membranes, and our immunofluorescence data also show a patchy cytoplasmic staining for PHD2, indicating an association with subcellular cytoplasmic structures (Fig. 1D). Additionally, it has recently been demonstrated that PHD2 interacts with the HIF-1α C-terminal ODD domain (aa 498 to 603) and thereby reduces the transcriptional activity of this fragment (44). Although we cannot exclude the possibility that attenuation of HIF-1α transcriptional activity by PHD2 is involved in reduced hypoxic HIF-dependent reporter gene (Fig. 5A) as well as HIF target gene (Fig. 6D) induction in FKBP38 siRNA clones, our data suggest that elevated PHD2 protein levels attenuate hypoxic HIF-1α stabilization (Fig. 5B and 6C).

PHDs represent attractive new therapeutic targets to modulate HIF activity, and thus functional understanding of PHD function and regulation is crucial. Recently, it has been reported that an inherited mutation in the active site of PHD2 leads to decreased hydroxylation activity and is associated with familial erythrocytosis (32). These data confirm previous studies with cultured cells implicating PHD2 as the primary PHD isoform responsible for HIF regulation under physiological conditions (2). Interestingly, a screen for genetic mutations in endometrial cancer cells identified PHD2 as being mutated at significantly higher frequencies (19). Expression of wild-type PHD2 in mutated cells induced senescence by negatively regulating HIF-1 expression, suggesting that PHD2 might be a candidate tumor suppressor. Specific upregulation of PHD2 in a FKBP38-dependent manner opens novel possibilities for interfering with one PHD family member specifically, thereby attenuating HIF signaling in cancer cells.

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CHAPTER THREE

Determination and Modulation of Prolyl-4-Hydroxylase Domain Oxygen Sensor Activity

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Contents

Abstract

The prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins hydroxylate hypoxia-inducible transcription factor (HIF)-alpha (α) subunits, leading to their subsequent ubiquitinylation and degradation. Since oxygen is a necessary

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cosubstrate, a reduction in oxygen availability (hypoxia) decreases PHD activity and, subsequently, HIF-α hydroxylation. Non-hydroxylated HIF-α cannot be bound by the ubiquitin ligase von Hippel-Lindau tumor suppressor protein (pVHL), and HIF-α proteins thus become stabilized. HIF-α then heterodimerizes with HIF-beta (β) to form the functionally active HIF transcription factor complex, which targets approximately 200 genes involved in adaptation to hypoxia. The three HIF-α PHDs are of a different nature compared with the prototype collagen prolyl-4-hydroxylase, which hydroxylates a mass protein rather than a rare transcription factor. Thus, novel assays had to be developed to express and purify functionally active PHDs and to measure PHD activity *in vitro*. A need also exists for such assays to functionally distinguish the three different PHDs in terms of substrate specificity and drug function. We provide a detailed description of the expression and purification of the PHDs as well as of an HIF-α–dependent and a HIF-α–independent PHD assay.

1. Introduction

Cells sense changes in environmental oxygen availability by a group of enzymes that directly control the cellular response to low oxygen by destabilizing HIF-α subunits, the master transcriptional regulators of the hypoxic response. These oxygen-sensing enzymes have alternatively been termed PHD, HIF prolyl hydroxylase (HPH), or egg-laying defective nine homolog (EGLN). Up to date, three family members are known: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1, and PHD3/HPH1/EGLN3 (Bruick, 2000; Epstein *et al.*, 2001; Ivan *et al.*, 2002). PHDs hydroxylate HIF-1α and HIF-2α at two distinct proline residues within the HIF-α oxygen-dependent degradation (ODD) domain (Fig. 3.1A). Under normoxic conditions, prolyl-4-hydroxylase allows binding of pVHL, leading to polyubiquitinylation and proteasomal destruction (Schofield and Ratcliffe, 2004; Wenger, 2002). Under hypoxic conditions, prolyl-4-hydroxylase is reduced, and HIF-1α and HIF-2α become stabilized, heterodimerize with the constitutively expressed HIF-1β subunit aryl hydrocarbon receptor nuclear translocator (ARNT), and regulate the expression of a large number of effector genes involved in adaptation to low oxygen (Wenger *et al.*, 2005). In addition, factor-inhibiting HIF (FIH) hydroxylates an asparagine residue within the C-terminal transactivation domain. Oxygen-dependent asparagine hydroxylation blocks the recruitment of the CREB-binding protein (CBP)/p300 transcriptional coactivators and thereby regulates the transcriptional activity of HIFs (Hewitson *et al.*, 2002; Lando *et al.*, 2002; Mahon *et al.*, 2001).

Prolyl-4-hydroxylase domain proteins do not represent static oxygen sensor molecules, but rather are highly regulated themselves. Importantly, PHD2 and PHD3, but not PHD1, have been reported to be hypoxically induced at both the messenger RNA (mRNA) and protein levels (Epstein

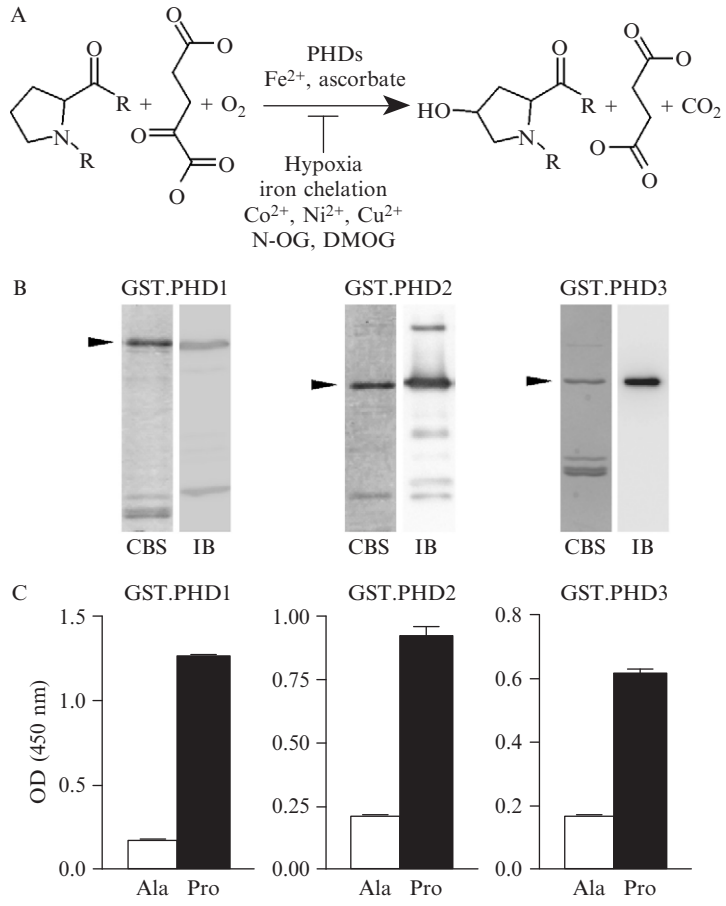

Figure 3.1 (A) Reaction mechanism of prolyl-4-hydroxylase domain (PHD)-mediated oxygen-dependent prolyl-4-hydroxylation by oxidative decarboxylation of the cosubstrate 2-oxoglutarate. Inhibitors of this reaction are indicated. N-OG, N-oxalylglycine; DMOG, dimethyloxalylglycine. (B) Purification of glutathione S-transferase (GST)-tagged PHD1, PHD2, and PHD3 expressed in baculovirus-infected *Spodoptera frugiperda* (Sf) 9 insect cells by glutathione affinity chromatography. The purified GST-PHD proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (CBS) or immunoblotting (IB) using anti-GST antibodies for the detection of GST-PHD1 and GST-PHD2 or anti-PHD3 antibodies for the detection of PHD3. (C) Hydroxylation activities of GST-PHD preparations determined by a von Hippel-Lindau tumor suppressor protein (pVHL)/elongin B/elongin C (VBC)-binding assay, as described in the text. Wild-type (Pro) and P564A mutant (Ala) hypoxia-inducible transcription factor (HIF)-1α oxygen-dependent degradation (ODD) domain-derived peptides were used as hydroxylation substrates.

et al., 2001). Accordingly, elevated PHD2 and PHD3 levels have been demonstrated in a broad panel of established cancer cell lines (Appelhoff *et al.*, 2004). Functional hypoxia response elements (HREs) are located in the promoter region of the human *PHD2* gene as well as in the first intron of the human *PHD3* gene, demonstrating that *PHD2* and *PHD3* are HIF target genes themselves (Metzen *et al.*, 2005; Pescador *et al.*, 2005). Because the essential cofactor oxygen is basically lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance is somehow paradoxical; it has been suggested that PHD induction plays a role in accelerating the termination of the HIF response following reoxygenation (Appelhoff *et al.*, 2004; Aprelikova *et al.*, 2004; Epstein *et al.*, 2001; Marxsen *et al.*, 2004). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the oxygen partial pressure (pO_2) in air, suggesting that the kinetics of specific HIF-α hydroxylation under hypoxic conditions are rather slow (Ehrismann *et al.*, 2006; Hirsilä *et al.*, 2003). Of note, these K_m values are critically dependent on the length of the target peptide (Koivunen *et al.*, 2006). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be "normoxic." We recently showed that HIF-dependent regulation of PHD levels adapts the PHD-HIF oxygen sensing system to a given tissue pO_2 rather than simply accelerating HIF-α destruction following reoxygenation (Stiehl *et al.*, 2006). Such a self-regulatory loop might define a tissue-specific threshold for HIF-α activation as a function of local pO_2.

In addition to transcriptional regulation, PHD levels are also regulated by protein–protein interactions: the ubiquitin ligase Siah2 regulates PHD1 and PHD3, but not PHD2, protein stability (Nakayama *et al.*, 2004); PHD3, but not PHD1 or PHD2, appears to be a substrate for the TRiC chaperonin (Masson *et al.*, 2004); OS-9 apparently is simultaneously interacting with both HIF-α and PHD2 or PHD3, but not PHD1, thereby enhancing HIF-α hydroxylation and degradation (Baek *et al.*, 2005); and Morg1 might provide the molecular scaffold for HIF-α interaction specifically with PHD3 (Hopfer *et al.*, 2006). On the other hand, PHD2 has been shown to inhibit the transactivation function of HIF-1α in VHL-deficient cells (To and Huang, 2005), a process that might involve PHD2-dependent recruitment of ING4, a likely component of a chromatin-remodeling complex (Ozer *et al.*, 2005).

These findings suggest two additional layers in oxygen signaling: first, abundance and function of PHDs actually can be regulated; and second, the three different PHDs are regulated in nonidentical ways, further supporting their non-redundant roles in oxygen sensing. In fact, while all three PHDs can hydroxylate HIF-α with similar efficiency *in vitro*, PHD2 has been suggested to play the main role for normoxic HIF-α turnover in cultured cells (Berra *et al.*, 2003). Consistent with these findings, PHD2, but neither PHD1 nor PHD3, knockout mice die during embryonic development (Takeda *et al.*, 2006). Interestingly, a family with erythrocytosis due to a

mutation in the gene encoding PHD2 has recently been reported (Percy *et al.*, 2006). The three PHDs are expressed in most organs; however, strikingly high levels of PHD3 and PHD1 mRNA are expressed in the heart and testis, respectively (Stiehl *et al.*, 2006; Willam *et al.*, 2006).

PHD function can also be regulated by a number of endogenous small molecules as well as by a number of clinically relevant drugs; ascorbate (Knowles *et al.*, 2003), transition metals (Hirsilä *et al.*, 2005; Martin *et al.*, 2005), Krebs cycle intermediates (Dalgard *et al.*, 2004; Lu *et al.*, 2005; Selak *et al.*, 2005), and reactive oxygen species (ROS), including NO (Berchner-Pfannschmidt *et al.*, 2006; Gerald *et al.*, 2004; Metzen *et al.*, 2003), have been shown to influence or completely block the activity of the PHDs. Thus, molecular cross-talks appear to exist between oxygen homeostasis and transition metal homeostasis as well as cellular metabolism. However, it is not completely understood how various transition metals and ascorbate interfere with PHD and FIH function. The transition metals do not simply replace or oxidize the ferrous iron in the active center of the hydroxylases (Hirsilä *et al.*, 2005; Martin *et al.*, 2005); at least cobalt and nickel might deplete intracellular ascorbate (Karaczyn *et al.*, 2006; Salnikow *et al.*, 2004). As known for collagen hydroxylation, ascorbate is essential in reducing ferric iron in the active center of PHDs and FIH, which occurs when oxidative decarboxylation of 2-oxoglutarate is uncoupled from target hydroxylation (McNeill *et al.*, 2005; Myllylä *et al.*, 1984).

In addition to kinase signaling pathways, numerous reports appeared about an involvement of ROS in HIF-α protein stabilization by either growth stimuli or hypoxia. Inconclusive data were obtained about the source(s) of the ROS and whether hypoxia leads to an increase or decrease in ROS levels (Wenger, 2000). However, ROS do have the potential to interfere with the complex process of protein hydroxylation. Indeed, the increase in ROS in *junD*$^{-/-}$ cells leads to a decrease in PHD activity and hence to HIF-1 α accumulation (Gerald *et al.*, 2004). Apart from direct interference of ROS with the active center of oxygen sensing protein hydroxylases, the redirection of oxygen from mitochondria towards the protein hydroxylases might contribute to these effects (Hagen *et al.*, 2003; Wenger, 2006).

Due to the large therapeutic potential of PHD inhibitors in the treatment of anemic and ischemic diseases, several attempts to develop PHD antagonists are in progress. Currently, N-oxyalylglycine (N-OG) and its cell-permeable derivative dimethyloxalylglycine (DMOG) are the commercially available PHD inhibitors of choice for experimental purposes. Apart from these cosubstrate competitive inhibitors, iron chelators are very well known to induce HIF by PHD inhibition. These include deferoxamine (DFX) and ciclopirox olamine (CPX), two clinically relevant hydroxamic acid iron chelators (Linden *et al.*, 2003). In order to functionally investigate the potency and mechanisms of available and future PHD inhibitors, a need exists for reliable methods to generate PHD proteins and to determine their activity.

2. Production of Functionally Active PHDs

Since bacterially expressed PHDs retain only little hydroxylase activity, PHDs were expressed and purified from baculovirally infected insect cells. To facilitate purification, the PHDs were tagged with either glutathione S-transferase (GST), maltose-binding protein (MBP), or His_6. According to our experience, best results were obtained with GST-tagged PHD proteins. Thus, GST-tagged expression vectors were prepared by LR clonase-mediated homologous recombination of the corresponding pENTR vector containing PHD1, PHD2, or PHD3 complementary DNA (cDNA) inserts, with the pDEST20 vector (Invitrogen, Carlsbad, CA). Recombined plasmids were transfected into the *Escherichia coli* strain DH10BAC, and the resulting bacmid plasmids were used to generate baculovirus stocks according to the manufacturer's instructions (Invitrogen). *Spodoptera frugiperda* (Sf) 9 cells were infected with baculovirus and cultured in Grace's insect medium (Invitrogen) at 27° in a humidified incubator. Infected cells were grown for 80 to 110 h, collected by centrifugation at 700×*g* for 10 min at 4°, and washed with ice-cold phosphate buffered saline (PBS). Lysis was performed on ice for 10 min with 0.1% NP-40 in a buffer containing 10 m*M* Tris-HCl, pH 7.4, 100 m*M* NaCl, 100 m*M* glycine, 100 μM dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA)-free complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Crude lysate was cleared by centrifugation at 20,000×*g* for 20 min at 4°, and the supernatant was incubated with glutathione-Sepharose beads (previously washed with PBS) for at least 2 h at 4° with gentle agitation. After washing three times with PBS, the protein was eluted with 15 m*M* reduced glutathione (GSH) in 50 m*M* Tris-HCl, pH 8.0, and 2 μM $FeSO_4$. Eluted proteins were supplemented with 5% glycerol and stored in aliquots at –80°. Purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining or immunoblotting (see Fig. 3.1B). Activity was routinely determined by the VHL binding assay described later (see Fig. 3.1C).

3. Determination of PHD Activity by VHL Binding to Peptides Derived from the HIF-1α ODD Domain

Enzymatic activities of recombinant PHD proteins were determined by an *in vitro* hydroxylation assay based on a 96-well format, as described previously (Oehme *et al.*, 2004). Briefly, biotinylated synthetic peptides derived from human HIF-1α amino acids 556 to 574

(biotin-DLDLEALAPYIPADDDFQL), either wild-type, P564A mutant, or hydroxylated (Hyp564), were bound to NeutrAvidin-coated 96-well plates (Pierce, Rockford, IL). All methionine residues were mutated to alanine residues in these peptides (M561A, M568A). Hydroxylase reactions using purified recombinant GST-tagged PHDs or cellular extracts were carried out for 1 h at room temperature. A polycistronic bacterial expression vector for His_6-tagged and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex (kindly provided by S. Tan, Pennsylvania State University, University Park, PA) was used to express VBC in *E. coli* strain BL21AI, followed by purification using nickel affinity chromatography (GE Healthcare, Buckinghamshire, UK), followed by anion exchange chromatography using a Hi Trap Q FF column (GE Healthcare) and gel filtration Hi Prep 26/10 desalting column; GE Healthcare). The VBC complex was allowed to bind to the hydroxylated peptides for 15 min, anti-thioredoxin antibodies were added for 30 min, and horseradish peroxidase-coupled anti-rabbit antibodies (Sigma, St. Louis, MO) were added for 30 min. Bound VBC complex was detected using the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit (Pierce). The peroxidase reaction was stopped by adding one volume 2 *M* H_2SO_4, and absorbance was determined at 450 nm in a microplate photometer. This assay is routinely used to determine the activity of the three GST-tagged PHD enzymes purified from Sf 9 insect cells (see Fig. 3.1C). Interassay comparability was guaranteed by calibration of each experiment to an internal standard curve using increasing fractions of synthetic hydroxyproline–containing peptides (Fig. 3.2A). When performed with pre-equilibrated solutions in a hypoxic glove box (Inviv02 400; Ruskinn Technology, Pencoed, UK), this assay demonstrates that PHD2 and PHD3 hydroxylase activities are functional over a wide range of physiologically relevant oxygen concentrations (see Fig. 3.2B). Furthermore, this assay is strictly dependent on the presence of P564 within the substrate peptide, and the iron chelator DFX as well as the substrate analog N-OG inhibit PHD-dependent prolyl-4-hydroxylation (see Fig. 3.2C).

Apart from purified recombinant PHDs, crude cellular extracts also are suitable sources of PHD activity for the VBC-binding assay. Therefore, Hep3B cells were lysed by dounce homogenization in 100 m*M* Tris-HCl, pH 7.5, 1.5 m*M* $MgCl_2$, 8.75% glycerol, 0.01% Tween20, and EDTA-free complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Cell lysates were cleared by centrifugation at 20,000×*g* for 30 min at 4°. These extracts stimulated VBC binding to wild-type, but not P564A mutant, HIF-1αODD-derived peptides (see Fig. 3.2D). The specificity of the PHD activity was further demonstrated by inhibition with 2 m*M* N-OG and by culturing the Hep3B cells under hypoxic conditions (0.4% O_2 for 16 h). Consistent with the known hypoxic induction of PHD2 and PHD3, extracts derived from hypoxic Hep3B cells showed an increased PHD activity (see Fig. 3.2D). Similar results were obtained with HeLa cells (data not shown).

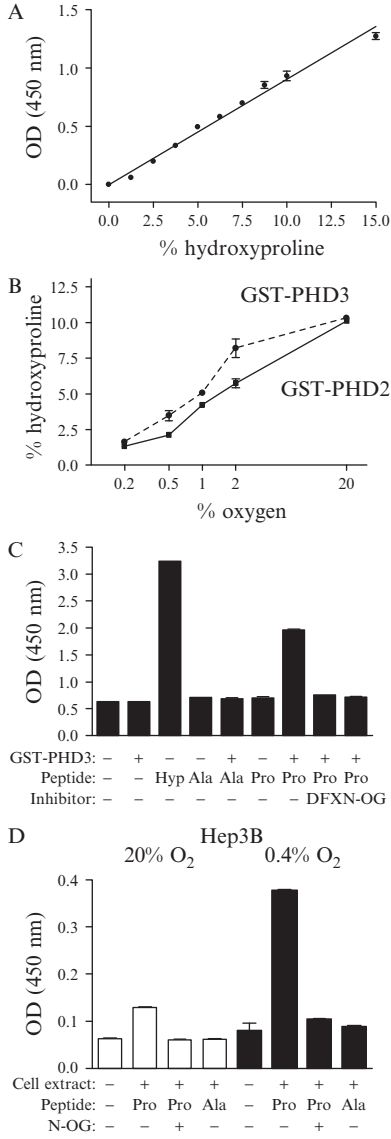

Figure 3.2 (A) Von Hippel-Lindau tumor suppressor protein (pVHL)/elongin B/elongin C (VBC) complex binding to prolyl-4-hydroxylated hypoxia-inducible transcription factor (HIF)-1α oxygen-dependent degradation (ODD) domain-derived peptides. Biotinylated peptides containing increasing proportions of synthetically generated hydroxyproline peptides were allowed to bind to NeutrAvidin-coated 96-well enzyme-linked immunosorbent assay (ELISA) plates. Immunodetection of bound VBC complex was performed, as described in the text. (B) Oxygen-dependent activity of recombinant glutathione S-transferase (GST) prolyl-4-hydroxylase domain (PHD) 2 and GST-PHD3. Purified PHD proteins were used to hydroxylate HIF-1α ODD

4. Determination of Prolyl-4-Hydroxylation by Oxidative Decarboxylation of 2-Oxoglutarate

While the VHL binding assay is well suited to investigate PHD function, it is strictly dependent on VBC binding and hence unlikely to investigate the hydroxylation of potential novel non–HIF-α targets. To overcome this problem, a 2-oxoglutarate to succinate conversion assay, originally developed to study collagen hydroxylation (Kaule and Günzler, 1990), was adapted to HIF-α hydroxylation. There are basically two possibilities to label the 2-oxoglutarate cosubstrate: when [5-^{14}C]2-oxoglutarate is used, the resulting [^{14}C]succinate can be measured (Kaule and Günzler, 1990); when [1-^{14}C]2-oxoglutarate is used, the resulting [^{14}C]CO_2 must be captured and quantified (Hirsilä *et al.*, 2003). Because it is technically less demanding, the former technique is used in our laboratory. This assay was first applied for PHD measurements by Frelin and coworkers using ''light mitochondrial rat kidney fractions'' (D'Angelo *et al.*, 2003a,b). We measured oxidative decarboxylation of 2-oxoglutarate by purified PHDs because kidney extracts prepared according to Frelin and coworkers solely showed high background activities, but did not contain measurable quantities of specific PHD activity (described later). Peptide (5–50 μM) or protein substrates (5 μM) were incubated with 0.1 μg GST-tagged PHD enzymes in 100 μl 50 mM Tris-HCl, pH 7.4, 10 μM 2-oxoglutarate, 2 mM ascorbate, 100 μM DTT, 1 mg/ml BSA, 0.6 mg/ml catalase, 5 μM freshly prepared $FeSO_4$, and 50,000 to 100,000 dpm [5-^{14}C] 2-oxoglutarate with a specific activity of 50 mCi/mmol (Hartmann-Analytic, Braunschweig, Germany). The 2-oxoglutarate concentration can be varied from 10 to 100 μM, depending on the peptide/protein target concentration and the desired specific radioactivity. Generally, a two-fold molar excess over the target concentration was used. Hydroxylation reactions were carried out for 24 h at 37°. To separate 2-oxoglutarate from succinate, 25 μl 20 mM succinate and 20 mM 2-oxoglutarate were added and mixed by vortexing. Subsequently, 25 μl 0.16 M 2,4-dinitrophenyl hydrazine in 30% $HClO_4$ were added. After incubation at room temperature for 30 min, 50 μl of 1M 2-oxoglutarate was added to remove residual 2,4-dinitrophenyl hydrazine, and the reaction was

domain-derived peptides under the indicated oxygen concentrations (%vol/vol in the gas phase). The extent of prolyl-4-hydroxylation was quantitated, as previously described. (C) PHD3 and the wild-type peptide substrate (Pro) are required for VBC binding. The lack of peptide (−), a P564A mutation (Ala), iron chelation by deferroxamine (DFX), or N-oxalylglycine (N-OG) all completely inhibited VBC binding. (A–C) Shown are mean values ± SEM of triplicates. (D) Hypoxia stimulates PHD activity in Hep3B cells. Crude cellular lysates were used to hydroxylate HIF-1α ODD domain-derived peptides, as previously described. Shown are mean values ± SEM of triplicates normalized to protein content.

allowed to proceed for another 30 min at room temperature. Following centrifugation at 20,000×*g* for 15 min at 4°, 150 μl supernatant was carefully removed, mixed with 3 ml scintillation cocktail (PerkinElmer, Waltham, MA), and the amount of [^{14}C]succinate was determined by liquid scintillation counting in a β-counter (TRI-CARB 2900TR, Packard).

As depicted in Fig. 3.3A, recombinant GST-PHD2 shows a linear relationship between the availability of an HIF-1α ODD domain-derived synthetic peptide substrate and [^{14}C]succinate production in the range from 8- to 50-μM peptide. Below 5 μM, however, only background values were obtained. This assay is strictly dependent on the presence of 1 mM ascorbate as well as a peptide substrate containing the wild-type P564 (see Fig. 3.3B).

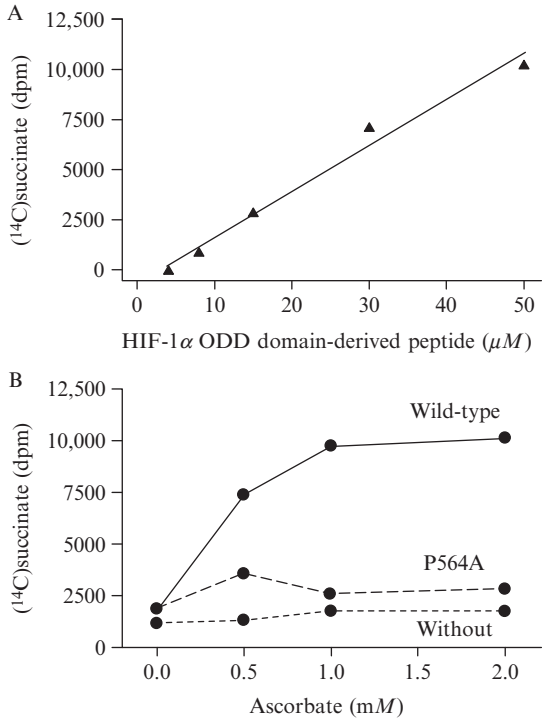

Figure 3.3 (A) Determination of prolyl-4-hydroxylation by oxidative decarboxylation of [5-^{14}C]2-oxoglutarate. Recombinant glutathione S-transferase (GST) prolyl-4-hydroxylase domain (PHD) 2 was incubated with increasing concentrations of a hypoxia-inducible transcription factor (HIF)-1α oxygen-dependent degradation (ODD) domain-derived peptide, as indicated. PHD-dependent generation of [^{14}C] succinate was determined, as described in the text. Background activity was determined in control reactions without substrate peptide and the obtained value subtracted from the peptide-containing reactions. (B) Recombinant GST-PHD2 was incubated with increasing concentrations of ascorbate (as indicated) and either a wild-type, P564A mutant, or no peptide substrate.

The lack of a substrate peptide or the mutation of the critical proline (P564A) results in background 2-oxoglutarate conversion.

5. Crude Tissue Extracts are not a Suitable Source of PHD Activity for the 2-Oxoglutarate Conversion Assay

Earlier reports suggested the use of "light mitochondrial rat kidney fractions" as a source for PHD activity in this type of assay (D'Angelo *et al.*, 2003a,b). We therefore prepared rat kidney extracts according to this protocol and tested them for PHD activity. As shown in Fig. 3.4A, 2-oxoglutarate conversion was indeed stimulated by these extracts. However, a very high background activity was also observed in the absence of the HIF-1α ODD domain-derived peptide, and the presence of the wild-type peptide stimulated 2-oxoglutarate conversion only moderately. To ensure PHD-dependent 2-oxoglutarate conversion, ample amounts of the known PHD inhibitors DFX and N-OG (see Fig. 3.2C) or $CuCl_2$ (Martin *et al.*, 2005) were added to the reaction mix. Surprisingly, none of these PHD inhibitors blocked 2-oxoglutarate conversion, whether the substrate peptide was present or not (see Fig. 3.4A, left panel). Moreover, the presence of a P564A mutant peptide stimulated 2-oxoglutarate conversion as well as the wild-type peptide (see Fig. 3.4A, right panel). The P564A mutant peptide cannot be hydroxylated and shows no VBC complex binding after incubation with purified PHDs (see Figs. 3.2C and 3.3B). Therefore, these experiments demonstrate that "light mitochondrial rat kidney fractions" do not represent a source of specific PHD activity, but rather contain high levels of enzymes not specifically metabolizing 2-oxoglutarate. A similar lack of specific (i.e., PHD-dependent) 2-oxoglutarate conversion was observed with crude extracts derived from HeLa, Hep3B, and Sf 9 cells (data not shown).

6. Thin Layer Chromatography to Assess the Purity of [5-^{14}C]2-Oxoglutarate

A major problem in the optimization of the 2-oxoglutarate conversion method is the high proportion of background activity when non-purified PHD preparations are used as a source of the enzymatic activity. Indeed, cellular extracts usually contain too high background activities to be suitable as PHD sources, even when the PHDs are exogenously overexpressed. Another background-causing problem was the quality of the radioactively labelled [5-^{14}C]2-oxoglutarate. To analyze the [5-^{14}C]2-oxoglutarate preparations for impurities, it was diluted in 1.5 m*M* unlabeled

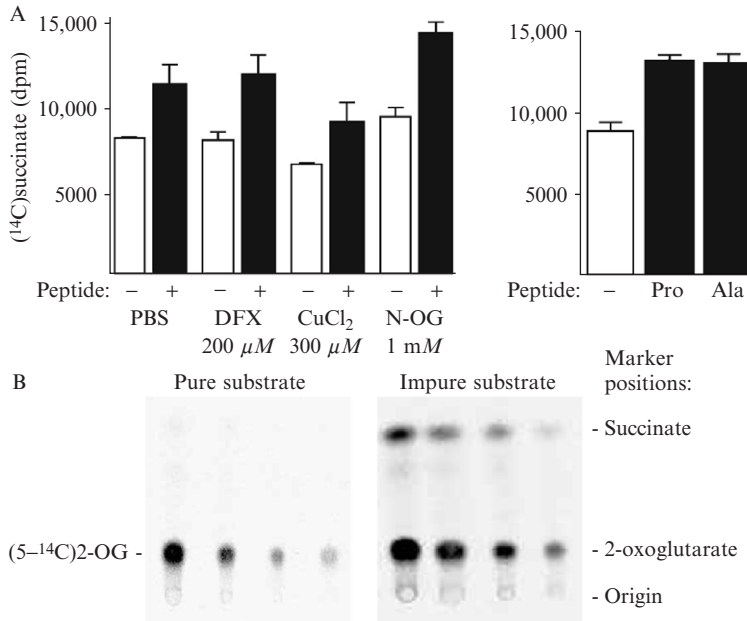

Figure 3.4 (A) Lack of specific 2-oxoglutarate-to-succinate conversion by "light mitochondrial rat kidney fractions." (Left panel) Peptide-stimulated activity in these extracts could not be inhibited by DFX, $CuCl_2$, or N-OG. (Right panel) A P564A mutant (Ala) hypoxia-inducible transcription factor (HIF)-1α oxygen-dependent degradation (ODD) domain-derived peptide stimulated 2-oxoglutarate conversion and the wild-type (Pro) peptide. (B) Quality control of [5-^{14}C]2-oxoglutarate by thin layer chromatography. The amount of [5-^{14}C]2-oxoglutarate spotted corresponded to 30, 15, 7.5, and 3.8 kdpm, respectively (from left to right). Two [5-^{14}C]2-oxoglutarate batches of different quality are shown. Pure 2-oxoglutarate and succinate served as migration markers (positions indicated in right margin).

2-oxoglutarate and spotted onto thin layer chromatography (TLC) plates (Silica gel 60 F254, Merck, Whitehouse Station, NJ). Following drying, the TLC plate was placed in a chromatography chamber containing a 120:70:15 mixture of diethyl ether/hexane/formic acid. When the eluent reached the top of the TLC plate, it was dried and the radioactivity quantitated by phosphorimaging (BioRad, Hercules, CA). Unlabeled 2-oxoglutarate and succinate were used as standards. They were visualized as bright yellow spots by immersing the TLC plates in a 0.04% bromcresol purple solution in a 1:1 mixture of ethanol and water (adjusted to pH 10.0 with NaOH) and dried with a hairdryer. R_f values for succinate and 2-oxoglutarate were 0.45 and 0.12, respectively. Considerable differences exist in the quality of the available [5-^{14}C]2-oxoglutarate preparations (see Fig. 3.4B). While some batches were of acceptable purity (Fig. 3.4B, left panel),

others contained up to 30% of an impurity that co-migrated with the succinate standard (Fig. 3.4B, right panel). Regarding the relatively low specific PHD activities and the rather high unspecific background 2-oxoglutarate conversion, such high impurities are not acceptable in this type of assay.

7. Application of the 2-Oxoglutarate Conversion Assay to Protein Targets

In order to be useful for putative novel PHD substrate proteins without prior knowledge of the actual target prolyl residue, the 2-oxoglutarate conversion assay needs to work also with proteins rather than only with synthetic peptides. To demonstrate the feasibility of this approach, wild-type GST-HIF-2αODD (aa 404–569, MW = 46 kDa) and P405A/P531A double-mutant GST-HIF-2αODD protein fragments were expressed in *E. coli* BL21AI by induction with 0.2% arabinose for 4 h at 37°. After harvesting by centrifugation, the bacteria were lysed with a high-pressure cell disrupter (Basic-Z, Constant Systems Ltd, Sanford, NC) in the presence of EDTA-free complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Purification of the GST-tagged protein was carried out using affinity chromatography on glutathione Sepharose beads (GE Healthcare, Buckinghamshire, UK), as previously described (see Fig. 3.1B). Purity of the recombinant protein fragments was checked by SDS-PAGE followed by Coomassie staining or immunoblotting (Fig. 3.5A). When compared with an HIF-1αODD–derived wild-type peptide, equimolar concentrations of the HIF-2αODD protein fragment also stimulated PHD1-dependent 2-oxoglutarate conversion, albeit to a somewhat lower extent (see Fig. 3.5B).

8. Conclusions

The PHD-dependent hydroxylation assay presented here works independently of already known peptide sequences or VHL binding. It should thus be possible to use this assay for the investigation of putative novel, non–HIF-α PHD substrates as well. In addition, even if not shown here, the same assay should principally be applicable to novel FIH substrates and might become useful for screening for novel hydroxylation targets. Whereas no non–HIF-α PHD hydroxylation targets have been reported thus far, FIH has recently been shown to hydroxylate a number of proteins containing

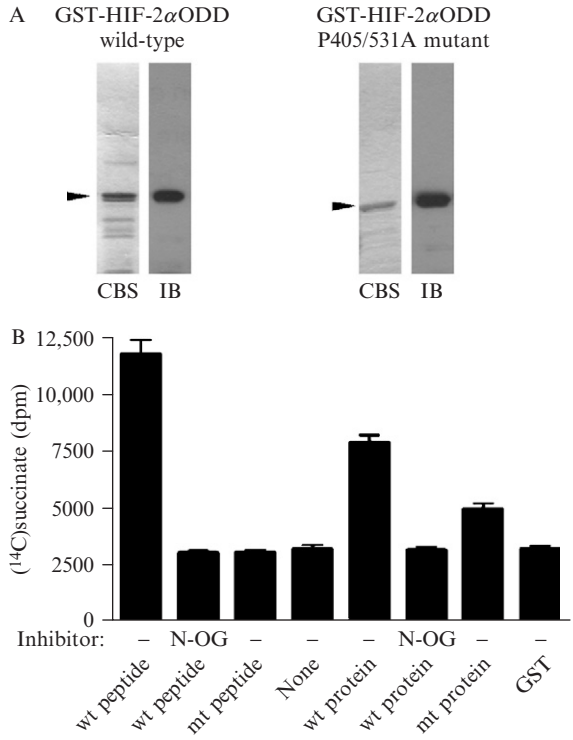

Figure 3.5 (A) Purification of wild-type and P405/531A double-mutant glutathione S-transferase (GST) hypoxia-inducible transcription factor (HIF)-2α oxygen-dependent degradation (ODD human HIF-2α amino acids 404–569) protein fragments expressed in bacteria and purified by glutathione affinity chromatography. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (CBS) or immunoblotting (IB) using anti-GST antibodies. (B) Determination of prolyl-4-hydroxylation by oxidative decarboxylation of [5-^{14}C]2-oxoglutarate. Recombinant GST-PHD1–dependent generation of [^{14}C] succinate was promoted by HIF-1α ODD-derived wild-type (wt), but not by P564A mutant (mt) peptides (human HIF-1α amino acids 556–574). Generation of [^{14}C]succinate was also promoted by wild-type GST-HIF-2α ODD protein, but not by GST alone and to a much lower extent by the GST-HIF-2α ODD P405/531A double mutant. Equimolar concentrations (5 μM) of the substrate peptides and protein fragments were used. Where indicated, the reactions could be blocked by 5 mM N-oxalylglycine (N-OG). Shown are mean values ± SEM of three independent experiments performed in duplicates.

ankyrin repeats, including NF-κB and IκBα (Cockman *et al.*, 2006). We expect a similar widening of the spectrum of PHD targets. In addition, PHD-dependent hydroxylation assays will be required to study novel drugs that modulate PHD activity and hence will become important for the treatment of anemic and ischemic disease.

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6 Manuscript

HIF PROLYL-4-HYDROXYLASE PHD2 PROTEIN STABILITY DEPENDS ON FKBP38 SUB-CELLULAR LOCALIZATION

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Running title: PHD2 protein stability depends on FKBP38 localization

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Prolyl-4-hydroxylases (PHDs) are oxygen-dependent enzymes that mediate the rapid destruction of the hypoxia-inducible factor (HIF)-α subunits. Three PHD isoforms (PHD1-3) are known to regulate HIF-α and they differ in their cellular and tissue distribution as well as in their *in vivo* functions as determined by genetic mouse models. PHD1 and PHD3 protein stability is regulated by the binding of the Siah1/2 ubiquitin ligase, followed by degradation in the 26S proteasome. However, little is known about PHD2 protein regulation. Recently, we reported that the FK506-binding protein 38 (FKBP38) specifically interacts with PHD2 and thereby modulates PHD2 protein stability in a peptidyl prolyl *cis/trans* isomerase (PPIase) independent manner. Here, we show that PHD2 protein regulation is independent of ubiquitinylation and proteasomal degradation but the FKBP38 transmembrane domain and therefore FKBP38 sub-cellular localization is essential for the interaction with PHD2 and required to determine PHD2 protein abundance.

Introduction

HIFs are central regulators of the cellular, local and systemic response to reduced oxygen partial pressure (pO_2). The HIF protein complex consists of a HIF-α and HIF-1β subunit (1), (2). HIF-α is constitutively expressed but under normoxic conditions rapidly degraded by the ubiquitin-proteasome pathway. HIF-α protein stability is determined by specific hydroxylation of prolines in the oxygen-dependent degradation domain (ODDD) by the oxygen-dependent PHDs and subsequent binding of the von Hippel-Lindau tumor suppressor protein (pVHL) (3), (4), (5), (5), (6). VHL is the recognition component of an E3 ubiquitin ligase complex and targets HIF-α for proteasomal destruction. So far, three PHD isoforms (PHD1-3) have been identified that mediate HIF-α protein stability in an oxygen-dependent manner (7), (8), (9). Additionally, the asparaginyl hydroxylase factor inhibiting HIF (FIH) regulates oxygen-dependently the transcriptional activity of HIF (10).

In hypoxia, PHDs and FIH are inactive and hence HIF-α accumulates, translocates to the nucleus and forms a heterodimeric complex with the constitutively expressed HIF-1β subunit. As a transcription factor, HIF regulates the expression of more than 70 target genes that allow the organism to adapt to hypoxia on the systemic, local and cellular level (11).

PHDs belong to the non-heme, Fe^{2+}- and 2-oxoglutarate-dependent family of dioxygenases. They require di-oxygen, Fe^{2+} and 2-oxoglutarate for their enzymatic reaction and therefore PHD activity correlates with the availability of oxygen. However, PHD2, PHD3 but not PHD1 are induced under hypoxia and are able to restrict hypoxic HIF response by compensating the lack of oxygen due to increased PHD protein levels and activity (12), (13). Besides HIF-α subunits, only a few other PHD hydroxylation targets have been described so far (14), (15), (16).

Interestingly, genetic ablation of PHD2 resulted in embryonic lethality between E12.5 to E14.5 due to defects in the developing placenta whereas *Phd1*-/- and *Phd3*-/- mice were viable (17). In adult mice, PHD2 somatic inactivation led to severe erythrocytosis as a result of increased erythropoietin (EPO) serum levels and EPO mRNA in the kidney whereas PHD1 and PHD3 single knockout mice showed no severe phenotype (18), (19). PHD1 and PHD3 double knockout animals demonstrated moderate erythrocytosis. *Phd1-/-* mice had a lowered oxygen consumption in skeletal muscle by shifting the glucose metabolism to anerobic glycolysis and preserved the muscle myofibers against ischemic necrosis (20).

Despite genetic ablation of PHD3 increased the number of superior cervical ganglion neurons in the sympathoadrenal system, the sympathoadrenal function was disturbed leading to low systemic blood pressure (21).

These genetic *in vivo* models suggest that PHDs might have redundant as well as non-redundant functions and seems conceivable that PHDs are itself a subject of regulatory mechanisms to limit their activity and/or stability.

Recently, we demonstrated that the FK506-binding protein 38 (FKBP38) specifically interacts with PHD2 but neither with PHD1 nor PHD3 (22). Stable downregulation of FKBP38 by RNAi in HeLa cells increased PHD2 protein abundance and therefore enhanced PHD hydroxylation activity and lowered HIF-1α accumulation under hypoxic conditions. Increased PHD2 protein levels resulted from enhanced PHD2 protein stability in a PPIase independent manner. Here, we report that the sub-cellular localization of FKBP38 is required for the regulation of PHD2 protein levels.

Experimental Procedures

Plasmids

If not indicated otherwise, cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland). Cloning of PCR fragments into entry vectors and recombination in destination vectors was described previously (22). All restriction enzymes were purchased from MBI Fermentas, Labforce, Nunnigen, Switzerland and New England Biolabs, United Kingdom. pENTR4-PHD2 from amino acid (aa) 1 to 162 (pENTR4-PHD2_1-162), pENTR4-PHD2_1-31, pENTR4-PHD2_1-58, pENTR4-PHD2_1-114 was obtained by amplifying PHD2_1-162, 1-31, 1-58, 1-114 from plasmid pENTR/D-PHD2 by PCR and subsequent cloning in *Xho*I/*Nco*I digested pENTR4-vector. pENTR/D-PHD2_21-426 and pENTR/D-PHD2_63-426 were obtained by digesting with *Msc*I/*Sca*I and *Msc*I/*Kas*I followed by Klenow fill-in and re-ligation. pENTR4-FKBP38_1-389 (ΔTM) was generated by insertion of a stop codon in pENTR4-FKBP38_1-412 by site-directed mutagenesis. The inserts of all entry vectors were verified by DNA sequencing (Microsynth, Balgach, Switzerland).

To generate fusion protein expression vectors, entry vectors were recombined *in vitro* with destination vectors using LR Clonase recombination enzyme mix (Invitrogen). The mammalian Matchmaker vectors pM and pVP16 (Clontech, BD Biosciences, Heidelberg, Germany) were converted to destination vectors by ligation of the Gateway vector conversion cassette reading frame B (Invitrogen) into the

*Eco*RI sites of pM and pVP16 to generate pM-DEST and pVP16-DEST, respectively. Expression vectors for Gal4 DNA-binding domain (pM) or VP16 activation domain (VP16) fusion proteins were obtained after *in vitro* recombination with the corresponding entry vectors. The mammalian one-hybrid plasmid pM-HIF-1α_370-429-VP16AD was generated described previously (22). Plasmids pDEST15 and pDEST17 were used to generate vectors for GST- and His_6-fusion protein expression in bacteria or in rabbit reticulocyte lysates. The plasmid used to generate recombinant GST-HIF-1α_530-826 was described previously (23). pDEST20 was used to generate expression vectors for GST-fusion proteins in the baculovirus/Sf9 insect cell system (Invitrogen). pcDNA3.1/nV5-DEST were used to express N-terminal V5-tagged proteins in mammalian cells, respectively. The mammalian fluorescent vectors pECFP-C1 and pEYFP-C1 (Clontech, BD Biosciences, Heidelberg, Germany) were converted to destination vectors by ligation of the Gateway vector conversion cassette reading frame B (Invitrogen) into the *Sma*I sites of pECFP-C1 and pEYFP-C1 to generate pECFP-C1-DEST and pEYFP-C1-DEST, respectively.

Chemicals

Chemicals were purchased from the following companies: CHX (Sigma Aldrich, Switzerland), E64 (Alexis Biochemicals), AEBSF (Alexis Biochemicals), MG132 (Sigma Aldrich, Switzerland), ALLM (Calbiochem), Pepstatin A (Alexis Biochemicals).

Peptide array synthesis

Using the standard SPOT synthesis protocol (24), the peptides were synthesized stepwise by an Abimed Asp 222 synthesizer on a cellulose membrane derivatized with two β-Ala residues as linker. 9*H*-fluoren-9-ylmethoxycarbonyl (Fmoc) protective groups were cleaved with 20% piperidine in dimethylformamide (DMF). After washing, the Fmoc amino acids (preactivated as pentafluorophenyl esters) were coupled. The side-chains of the amino acids were protected as follows: Pbf (arginine), Trt (asparagine, glutamine, histidine, cysteine), tBu (threonine, tyrosine, serine), OtBu (aspartic acid, glutamic acid), Boc (lysine, tryptophan). After coupling, all unreacted amino groups were blocked with acetic anhydride. In the final cycle, the deprotection with piperidine was performed before acetylation and thus N-terminal acetylated peptides were obtained. All protective groups were cleaved for 3 h with 30 ml 50% trifluoroacetic acid (TFA) / dichlormethane (DCM) containing 1 ml

triisopropylsilane (TIPS).

Western blot analysis of PHD2 interaction with the peptide array

Before Western blot screening, the dry peptide-array membranes were rinsed for 10 min in methanol and for 3x20 min in TBS buffer (30 mM Tris/HCl, pH 7.6, 170 mM NaCl, 6.4 mM KCl). PHD2 variant solutions (100 nM) in TBS buffer were allowed to react with peptide-array membranes for 4 h at 4°C under gentle shaking. The membrane was subsequently washed 3 times with TBS buffer, before bound protein was blotted onto nitrocellulose membranes and analyzed using polyclonal rabbit anti-PHD2 antibodies (Abcam, Cambridge, United Kingdom).

Peptide synthesis

The peptides were produced by solid-phase peptide synthesis with the robot Syro II (MultiSynTech, Witten, Germany) using 0.15 mmol pre-loaded Fmoc-Asn(Trt)-Wang resin from NovaBiochem (Läufelfingen, Switzerland). The synthesis was performed by Fmoc strategy and standard protocol, with Fmoc amino acids as building blocks, as well as benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), (NovaBiochem) and N-methyl-morpholine as coupling reagents in dimethylformamide (DMF). Piperidine (20%)/DMF was the standard cleavage cocktail used for Fmoc detachment. The resin was treated twice for 10 minutes. All couplings were performed using a 4-fold excess of Fmoc amino acid derivative, PyBOP and N-methyl-morpholine in DMF. A double coupling protocol was used. The coupling of biotin at the N-terminal position of the protected peptide was carried out in N-methyl-pyrrolidone/DMSO (1:1 v/v). After detachment of the peptides from the resins and side-chain deprotection with TFA/TIPS/thioanisol/N-(methyl) mercaptoacetamide/ water (80/7.5/5/2.5/2.5/2.5 v/v) at room temperature for 12 h, the crude peptides were precipitated with diethylether and purified by RP-HPLC using Gilson 306 equipment with a SP 250/10 Nucleosil 100-7 C8 column (Macherey-Nagel, Düren, Germany) by applying a water (0.1% TFA/acetonitrile) gradient. The purified peptides were lyophilized. The purity of the peptides was evaluated by analytical HPLC and the correct molecular masses were confirmed by MALDI-TOF mass spectrometry.

Protein binding assay

Streptavin agarose (Sigma, Deisenhofen, Germany) was saturated with Biotin-EEEEEEEEEEDDLSELPPLE-NH2 peptide and washed three times with incubation buffer (25 mM Tris/HCl, 200 mM NaCl, 1 mM DTT, pH 7.5). PHD2 variants (1 µM)

were incubated either in presence or absence of Ac-EEEEEEEEEEDDLSELPPLE-NH2 peptide (20 μM) and FKBP38 (10 μM) with the affinity matrix for 1 h at 4 °C. Subsequently, the samples were washed three times with incubation buffer, boiled in Laemmli buffer and subjected to SDS-PAGE. Binding of PHD2 was analyzed using polyclonal rabbit anti-PHD2 antibodies (Abcam, Cambridge, United Kingdom).

For incubation with endogenous rat proteins, rat liver was decomposed in 50 mM Hepes buffer (4% Chaps, 1% DTT, 100 mM NaCl, 0.1% NaN_3, pH 7,5) and centrifuged at 14'000 *x g* for 10 min at 4°C. The supernatant was applied to the affinity matrix in analogy to the PHD2 proteins.

Fluorescence spectroscopy

Steady-state fluorescence spectra were recorded on a Perkin Elmer FluoroMax2 fluorescence spectrometer, using a 1 × 1 cm cuvette with an excitation wavelength of 280 nm and excitation and emission slit widths of 5 and 3 nm, respectively. To compensate for inner filter effects, the samples for fluorescence measurements were diluted to an optical density at 280 nm of 0.15. Protein samples were applied in 25 mM Tris/HCl buffer (200 mM NaCl, 1 mM DTT, pH 7.5). The binding constant (K_D) was calculated from the fluorescence intensity by using the equation:

$$P_0 \cdot \alpha = \frac{C_0 \cdot \alpha}{n(1-\alpha)} - \frac{K_D}{n}$$

where P_0 = total protein concentration, $\alpha = (F_{max} - F)/(F_{max} - F_0)$, F_{max} = fluorescence intensity at saturation, and F_0 = initial fluorescence intensity, n = number of independent binding sites, C_0 = total PHD2 concentration at each addition, and K_D = dissociation constant.

Protein expression and purification

GST and GST-fusion proteins were expressed in *E. coli* BL21-AI by induction with 0.02% arabinose for 4 h and purified using glutathione sepharose beads (Amersham Biosciences). Purification of GST-PHD2 from Sf9 insect cells was previously described (25).

In vitro transcription/translation (IVTT) and GST pull-down

IVTT reactions were carried out as described by the manufacturer instructions (Promega, Madison, WI, USA) using recombined destination vectors in the presence of ^{35}S-Met (Hartmann Analytic, Braunschweig, Germany). Purified GST-tagged proteins (10 μg) were diluted in bead binding buffer (50 mM potassium phosphate pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1% TX-100) and incubated with

glutathione sepharose beads. For pull-down experiments, 20 μl rabbit reticulocyte IVTT reaction were incubated for 2 h at 4ºC with bound GST-fusion proteins in Co-IP buffer containing 50 mM Tris-HCl pH 7.6, 2 mM EDTA, 100 mM NaCl, 0.1% TX-100, washed 4 times with Co-IP buffer, boiled in sample buffer (40 mM Tris-HCl pH 6.8, 1% SDS, 50 mM β-mercaptoethanol) for 5 min and separated by SDS-PAGE. Gels were stained with coomassie blue, dried and radioactively labeled proteins detected by phosphorimaging (Molecular Imager FX, BioRad).

Cell culture and transient transfection

The mouse E1-ubiquitin-activating enzyme temperature sensitive ts20 cells and the ts20 cell line reconstituted with wild-type E1 gene (H38-5) were kindly provided by Prof. C. Borner (Insitute for Molecular Medicine and Cell research, University of Freiburg, Freiburg, Germany). The mouse primary FKBP38 knockout and wild-type embryonic fibroblasts (MEFs) were a kind gift of M. Shirane (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). HeLa cervical carcinoma, HEK293 embryonic kidney carcinoma, MCF-7 breast cancer, mouse ts20, H38-5, wild-type and MEF FKBP38 knockout cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) as described previously (15). Transient transfections were performed with the polyethylenimine (PEI; Polysciences, Warrington, PA, USA) method (12).

Western Blot analysis

Total cell extracts of cultured cells were prepared using RIPA buffer containing 50 mM Tris Cl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS. Protein concentration was determined using the BCA assay (Pierce, Perbio Science, Lausanne/Switzerland). Immunoblotting was performed as described previously (26). Briefly, protein was separated by SDS-PAGE, electro-transferred onto nitrocellulose membranes (Amersham Biosciences) and incubated with antibodies. The following antibodies were used: rabbit anti-PHD2 (Novus, Abcam, Cambridge. United Kingdom), rabbit anti-mouse PHD2 (Novus Biological), rabbit anti-FKBP38 (27), mouse anti-β-actin (Sigma Aldrich, Switzerland), mouse anti-PDI (Novus Biological), rabbit anti-Mitofilin (Novus Biological), mouse anti-p53 (Santa Cruz Biotechnology) and mouse anti-V5 antibody (Invitrogen).

Fluorescence microscopy and fluorescence resonance energy transfer (FRET) analysis

Cells were cultivated on microscope slides, washed twice with ice-cold PBS, fixed on ice for 30 min with 4% paraformaldehyde and permeabilized with 0.1% saponine in PBS. Endogenous as well as transfected proteins were detected using the indicated antibodies. Cellular organelles were stained with Mitotracker (Molecular Probes, Invitrogen), WGA-Alexa 594 (Molecular Probes, Invitrogen) and with antibodies against PDI or Calreticulin (Novus Biologicals). Immune complexes were visualized with goat anti-rabbit Alexa 488, goat anti-mouse Alexa 488, goat anti-rabbit Alexa 568 or goat anti-mouse Alexa 568 (Molecular Probes, Invitrogen), respectively. Nuclei were stained with DAPI (Sigma Aldrich, Switzerland) for 30 min. After extensive washings with PBS, the microscope slides were embedded in Mowiol and analyzed by confocal laser scanning microscopy (SP1, Leica Microsystems, Switzerland). For FRET analysis, HEK293 cells were transfected with indicated plasmids and FRET was monitored as described previously (28).

Mammalian one- and two-hybrid assays

Mammalian one- and two-hybrid analyses were performed using the mammalian Matchmaker system (Clontech, BD Biosciences) described previously (22). HeLa cells were transiently co-transfected with 10 ng one-hybrid or 1.5 μg Gal4 DNA binding domain (DBD) and 1.5 μg Gal4 activation domain (AD) fusion protein vectors, together with 500 ng of firefly luciferase reporter vector pGRE5xE1b and 20 ng pRL-SV40. Luciferase reporter gene activity was determined using the dual luciferase reporter assay system according to the manufacturer's instructions (Promega).

Sub-cellular fractionation

Cells were incubated in hypotonic buffer (10 mM HEPES pH 7.5, 1 mM EGTA, 25 mM KCl) on ice for 20 min and afterwards dounce homogenized in homogenization buffer (10 mM HEPES pH 7.5, 1 mM EDTA, 250 mM sucrose). Cell lysates were centrifuged for 15 min at 4ºC at 3'000 *x g* to pellet nuclei and non lysed cells. To separate the membrane from the cytosolic fraction the supernatant was centrifuged for 1 h at 4ºC at 100'000 *x g*. The cytosolic fraction was stored for immunoblotting. The membrane fraction was resuspended in homogenization buffer and overlayered above a 10 to 30% iodixanol gradient and centrifuged for 18 h at 150'000 *x g* to

separate the membrane fractions. By punctuation of the centrifugation tubes, 1 ml fractions were obtained and analyzed by immunoblotting.

In vitro VBC-dependent prolyl-4-hydroxylation assay

$1x10^8$ HeLa cells were pelleted and dounce homogenized in 1 ml cell lysis buffer (100 mM Tris-Cl pH 7.5, 1.5 mM $MgCl_2$, 8.75% glycerol, 0.01% Tween 20) supplemented with EDTA free complete protease inhibitors (Roche). Lysates were centrifuged for 30 min at 4°C at 20'000 *x g* and VBC-binding was determined as previously described (29), (25).

RNA extraction and real-time RT-qPCR quantification

Cells were grown in 15 cm plates under normoxic or hypoxic conditions and total RNA was extracted as described previously (26). First strand cDNA synthesis was performed with 3 μg of RNA using AffinityScript^{TM} multiple temperature reverse transcriptase (RT) according to the manufacturer's instructions (Stratagene). Subsequently, mRNA expression levels were quantified with 2 μl of diluted cDNA reaction by real-time PCR using a SybrGreen Q-PCR reagent kit (Sigma) and the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, mouse ribosomal protein S12 mRNA was determined and data expressed as ratios relative to mS12 levels. Primers were used as follows: mS12 forward 5'-gaagctgccaaagccttaga-3', mS12 reverse 5'- aactgcaaccaaccaccttc-3' mFKBP38 forward 5'-attgctggcaggaactgact-3', mFKBP38 reverse 5'-cagtccttcctgcttcttgg-3' mPHD1 forward 5'-ttgcctgggtagaaggtcac-3', mPHD1 reverse 5'-gctcgatgttggctaccact-3'; mPHD2 forward 5'-gcaacggaacagg6ctatgtc-3', mPHD2 reverse 5'-ctcgctcatctgcatcaaaa-3'; mPHD3 forward 5'-caacttcctcctgtccctca-3', mPHD3 reverse 5'-ggctggacttcatgtggatt-3'.

Results

Biochemical characterization of the PHD2:FKBP38 interaction.

Recently, we reported that FKBP38 interacts with PHD2 but neither with PHD1 nor PHD3 (22). To further characterize the interacting domains of both proteins, we performed a peptide scan. A peptide array of 13mer peptides covering the entire protein sequence of FKBP38 was incubated with purified PHD2. PHD2 bound to peptides corresponding to FKBP38 from aa 37 to 56 to the N-terminal extension of

FKBP38, preceding the catalytic domain (Fig. 1A upper panel). PHD2 did not bind the reverse sequence of this motif in the peptide array demonstrating the stereo specificity of the interaction between PHD2 and the FKBP38_37-56 motif (Fig. 1B lower panel). In contrast, an N-terminal truncated PHD2 protein from aa 170 to 426 did not bind to the peptide array, demonstrating the requirement of the N-terminal PHD2 domain for the interaction with FKBP38 (data not shown).

Based on these results, the peptide biotinyl-EEEEEEEEEEDDLSELPPLE-NH_2, which corresponds to the PHD2-interacting FKBP38 motif, was immobilized on streptavidin beads and subsequently analyzed for binding to purified PHD2 protein. PHD2 bound to the affinity matrix and this binding was diminished in the presence of a peptide corresponding to PHD2-binding motif of FKBP38 or FKBP38 itself, indicating competition between matrix and soluble FKBP38 variants for PHD2 binding (Fig. 1B). In contrast, no binding of PHD2 170-426 to the affinity matrix was observed. These results suggest that the N-terminal PHD2 domain binds specifically to the identified motif in the FKBP38 N-terminus. In a further attempt, rat liver lysate was applied to either the affinity matrix or streptavidin beads done to test whether endogenous PHD2 interacts with the binding motif in FKBP38. Endogenous rat PHD2 bound only to the affinity matrix (Fig. 1C).

To further analyze the interaction between FKBP38 and PHD2, measurements of the protein fluorescence of both interaction partners were performed. Upon the interaction between the N-terminal domains of FKBP38 and PHD2 the fluorescence spectrum was blue-shifted by 2 nm and the amplitude was increased by 12% compared to the sum of the fluorescence spectra of the separated proteins (Fig. 2A). In comparison, no changes in the protein fluorescence were observed, when the PHD2_170-426 was added to FKBP38 (Fig. 2B), again demonstrating the requirement of the N-terminal PHD2 domain for the interaction with FKBP38. Interestingly, the increase in the fluorescence signal, which occurs upon FKBP38:PHD2 interaction of the full-length proteins, was reduced in presence of the peptide corresponding to the PHD2-binding site in FKBP38 in a concentration dependent manner (Fig. 2C). The measurements resulted in a K_D value of 1.48 ± 0.15 µM for the interaction between FKBP38-37-56 peptide and PHD2.

Furthermore, we performed isothermal titration calorimetry (ITC) measurements to characterize the interaction between PHD2 and the N-terminal extension of FKBP38 (data not shown). The titration curve revealed a 1:1 complex and a dissociation

constant (K_D) of 895 ± 148 nM for this interaction. For interactions between PHD2 and FKBP38_37-56 ITC measurements showed as well a 1:1 stoichiometry and a K_D = 1.28 ± 0.19 µM, which is similar to the result of the fluorescence titration curve.

Mapping PHD2 interaction domain in FKBP38.

PHD2 consists of a N-terminal MYND-type Zn^{2+} finger domain from aa 21 to 58 and a prolyl-4-hydroxylase catalytic subunit from aa 205 to 391 (Fig. 3A). Previously, we have shown that FKBP38 bound to the N-terminal region of PHD2 from aa 1 to 170. To further investigate the detailed interaction domain, we generated several PHD2 N-terminal deletion constructs and analyzed the interaction in GST pull-down experiments (Fig. 3A). GST-FKBP38 strongly interacted with *in vitro* transcribed and translated (IVTT) ^{35}S-labeled full-length PHD2 and PHD2_1-162 but no interaction was observed when GST-FKBP38 was incubated with any of the N-terminal deletions PHD2_21-426, 63-426 and 170-426 (Fig. 3B). GST alone did not bind to the different PHD2 fragments. We then generated several PHD2 C-terminal deletion fragments tested them for *in vitro* interaction. Surprisingly, IVTT ^{35}S-labeled FKBP38 only bound to GST-PHD2_1-114 but not to GST-PHD2_1-31, GST-PHD2_1-58 (Fig. 3C and D). Therefore, we propose an interaction of FKBP38 with the N-terminal region of PHD2 from aa 1 to 114 containing the MYND-type Zn^{2+} finger domain. In addition, experiments investigating the binding of recombinant FKBP38 to a PHD2 13mer peptide array did not lead to the identification of a distinct binding domain, suggesting a non-linear FKBP38 binding region in PHD2 (data not shown).

PHD2 protein abundance is elevated in Fkbp38-/- cells.

FKBP38 knockout mice die around embryonic day E13.5 due to defects in the development of the central nervous system (30). However, in another genetic background knockout of FKBP38 did not affect the viability of the mice but these mice displayed severe bone and cartilage deformation, probably due to increased cell death in the posterior neural tube (31). In HeLa FKBP38 knockdown cells, PHD2 protein abundance was increased and PHD2 half life was prolonged whereas PHD2 mRNA was not affected (22). We measured PHD2 protein and mRNA levels in primary *Fkbp38*+/+ and *Fkbp38*-/- MEF cells. Comparable to FKBP38 downregulation in HeLa cells, PHD2 protein levels were slightly increased in normoxia (Fig. 4A). Under hypoxic conditions PHD2 protein was less induced compared to *Fkbp38* wild-type MEFs (Fig. 4A). Neither PHD1 nor PHD2 and PHD3 mRNA levels were affected in *Fkbp38* knockout MEFs compared to wild-type MEFs

in normoxia (Fig. 4B).

PHD2 protein stability is regulated in an ubiquitin-independent manner.

To explore the molecular mechanism of FKBP38 mediated PHD2 protein regulation, we investigated the proteolytic destruction of PHD2. MCF-7 cells were incubated in normoxia with protease inhibitor E64 (cysteine protease inhibitor), AEBSF (serine protease inhibitor), Pepstatin A (aspartyl protease inhibitor), ALLM (calpain inhibitor, catepsin L, B and neutral cysteine protease inhibitor) and MG132 (proteasomal inhibitor). Additionally, MCF-7 cells were pre-incubated for 24 h under hypoxic conditions and re-oxygenated in the presence of the described protease inhibitors above. Neither under normoxic conditions (Fig. 5A) nor during re-oxygenation (Fig. 5B) PHD2 protein levels were affected by the incubation with these protease inhibitors. PHDs have been described to interact with the ubiquitin ligase Siah1/2 (32). However, the functional consequence of the PHD2:Siah1/2 interaction remained unknown. To completely exclude the possibility of ubiquitin-mediated PHD2 proteasomal destruction we made use of mouse ts20 cells that possess a temperature sensitive E1-ubiquitin activating enzyme (33). Mouse PHD2 protein abundance was not affected in ts20 cells incubated under 34ºC or 39ºC for up to 48 h (Fig. 5C). Additionally, no changes were observed for FKBP38. However, p53 a known protein target to be degraded by the ubiquitin-proteasome system, strongly accumulated under 39ºC incubation. As control, incubation of a ts20 cell line stably transfected with a wild-type E1 gene (H38-5) under 34ºC or 39ºC did not result in the accumulation of PHD2, FKBP38 and p53 (Fig. 5D). Furthermore, we questioned if different oxygen concentrations might influence PHD2 protein half-life. Hence, HeLa cells were treated with 100 μM CHX for 24, 48 and 72 h and cultivated either at 20% O_2 or under 0.2% O_2 (Fig. 5E). PHD2 protein stability was not significantly affected by the different oxygen concentration. Additionally, FKBP38 also did not show any oxygen-dependent differences in the protein stability.

FKBP38 transmembrane domain determines PHD2 protein abundance.

FKBP38 has been described to localize to the mitochondria and endoplasmatic reticulum (ER) (30) and we questioned whether the sub-cellular localization of FKBP38 might be important for PHD2 protein regulation. Hence, we generated a FKBP38 expression plasmid where the C-terminal transmembrane domain of FKBP38 (FKBP38_1-389) was deleted. Transiently transfected full length V5-tagged FKBP38_1-412 and FKBP38Δ98-257 lacking the PPIase domain localized to

mitochondria (mitotracker) and endoplasmatic reticulum (calreticulin) (Fig.6A and B). FKBP38_1-389 was expressed diffusively throughout the cytosol and did not co-localize either with Mitotracker or with calreticulin (Fig.6A and B). All V5-FKBP38 fusion proteins did not localize to the golgi apparatus stained with WGA (Fig. 6C). Changes in the PHD2 protein levels directly influence the stability of HIF-α subunits. To investigate the stability of HIF-1α we co-transfected MCF-7 cells with HIF-1α_370-429 flanked at the N-terminal end with Gal4-DBD and at the C-terminal end with VP16AD, a firefly luciferase reporter gene construct containing five Gal4-DBD responsive elements and a *Renilla* luciferase control plasmid. As shown in figure 6D expression of V5-tagged FKBP38_1-412 and FKBP38Δ98-257 enhanced greatly HIF-1α one-hybrid stability compared to mock transfected MCF-7 cells, but the effect was not observed by FKBP38_1-1389 (ΔTM) expression in normoxia and hypoxia. All expression plasmids were equally expressed as shown in figure 6D. Similar results were obtained in HeLa cells stably transfected either with a control plasmid harboring nonspecific oligonucleotides (ctrl) or a FKBP38-silencing construct (3D6, 2G8). Expression of full length FKBP38 strongly increased the HIF-1α 370-429 one hybrid stability and it was comparable to mock transfected cells when FKBP38 1-389 was overexpressed (Fig. 6E). Of note, in the FKBP38 knockdown cells the HIF-1α one hybrid construct is less stabilized than to control cell line due to increased PHD2 protein stability under normoxic and hypoxic conditions. Furthermore, PHD2 protein levels were increased in HeLa FKBP38 depleted cell lines 3D6 and 2G8 compared to control and wild-type HeLa cells (Fig. 6F). Overexpression of full length V5-tagged FKBP38 in HeLa 3D6 and 2G8 reduced PHD2 protein levels comparable to control and wild-type HeLa cells wheras overexpression of V5-FKBP38_1-389 did not affect PHD2 protein levels (Fig. 6F), suggesting that the transmembrane domain of FKBP38 might be required in regulating PHD2 protein abundance. Consistent with these data, increased hydroxylation activity was observed in the FKBP38-depleted cells by using the VBC-dependent prolyl-4-hydroxylase assay (34), (25) and only expression of V5-tagged FKBP38_1-412 but not FKBP38_1-389 normalized prolyl hydroxylation (Fig. 6G).

Correct FKBP38 sub-cellular localization is required for interaction with PHD2.

To investigate whether the interaction of FKBP38 with PHD2 depends on the sub-cellular localization of FKBP38, we applied FRET technology. We observed a FRET signal when CFP-FKBP38 and YFP-PHD2 were expressed in HEK293 cells. As

control, no signal was detected when the interaction domain of FKBP38 (CFP-FKBP38_99-412) was deleted (Fig. 7A). Surprisingly, no FRET signal was observed when CFP-FKBP38_1-389 (ΔTM) was co-expressed with YFP-PHD2 despite of the presence of the interaction domain of FKBP38 (Fig. 7A). However, in *in vitro* GST pull-down experiments GST-tagged FKBP38_1-412 and GST-FKBP38_1-389 interacted with ^{35}S-labeled PHD2 (Fig. 7B). To confirm the requirement of the FKBP38 transmembrane domain for the interaction with PHD2 in cells, we applied mammalian two-hybrid technology. The activity of a co-transfected luciferase reporter gene construct is greatly enhanced when DBD-PHD2 and AD-FKBP38_1-412 were co-transfected in HeLa cells (Fig. 7C). Consistent with the FRET data, low luciferase reporter gene activity was measured when DBD-PHD2 was co-expressed with AD-FKBP38 1-389. Co-transfection of DBD-p53 with AD-CP or with AD-LT served as a positive and negative control, respectively. These data suggest that the transmembrane domain of FKBP38 is required for the binding to PHD2 in a cellular context.

PHD2 co-localizes with FKBP38 in the cellular membrane fraction.

By overexpression studies, PHD2 has been reported to be localized mainly in the cytosol (35). However, detailed sub-cellular localization of PHD2 has not been described so far. FKBP38 has been reported to be a membrane associated protein localized in the ER and in mitochondria (30). Since the localization of FKBP38 seems to be important for regulation of PHD2 protein amount, we investigated the sub-cellular localization of PHD2 in more detail. By biochemical fractionation we analyzed PHD2 protein levels in the membrane and cytosolic fractions of wild-type HeLa cells and the FKBP38-knockdown cell line 2G8 (Fig. 8A and B). We observed an increase in PHD2 protein amount with decreased FKBP38 protein levels in the FKBP38 depleted cells but no shift of PHD2 protein in dependency of FKBP38 availability from the membrane to cytosolic fraction was seen (Fig. 8B). Furthermore, cells were incubated for 16 h in hypoxia and PHD2 protein amount was detected in the membrane as well as cytosolic fraction in both cell lines, however to a higher extend in the FKBP38-depleted cells (Fig. 8C-E).

Discussion

HIF-α protein stability is tightly controlled by hydroxylation of specific prolines in the ODDD by the oxygen-sensing prolyl-4-hydroxylases PHDs. PHDs are not only

regulated by the availability of oxygen, as PHD2 and PHD3 expression is transcriptionally induced by HIF under hypoxic conditions and thereby compensating reduced oxygen partial pressure (pO_2) (12), (13). PHD1 and PHD3 protein abundance are regulated by binding to the E3 ubiquitin ligase Siah1/2 and both proteins are targeted for proteasome-dependent degradation (32). Additionally, PHD2 interacted with Siah1/2 though the functional consequence remained elusive. Recently, we reported that PHD2 protein abundance is regulated by the peptidyl prolyl *cis/trans* isomerase FKBP38 in a peptidyl prolyl *cis/trans* isomerase-independent manner (22). In this present study, we characterized in more detail the functional consequence of the FKBP38:PHD2 interaction. We discovered that the PHD2:FKBP38 interaction did not only require the N-terminal binding motif of FKBP38 but in addition the transmembrane domain of FKBP38 was essential for the interaction with PHD2. Moreover, the FKBP38 transmembrane domain and therefore FKBP38 sub-cellular localization was crucial for PHD2 protein regulation.

FKBP38 binds with a minimal linear glutamate-rich binding motif from aa 37 to 56 to PHD2, a so far unknown interaction domain. PHD2 interacts with a non-linear motif from aa 1 to 114 to FKBP38. This region contains a MYND-type Zn^{2+} finger domain. A large group of proteins among the vertebrates have a MYND-type Zn^{2+} finger domain, mainly functioning as an interaction domain to recruit transcriptional co- and repressor complexes. PHD2 has been reported to have a potential transcriptional activity (36). Additionally, PHD2 has been demonstrated to interact with ING4 to recruit transcriptional repressors to limit the HIF response (37). We found a specific interaction of the N-terminal region of PHD2 with FKBP38. FKBP38 localization has been described to mitochondria and endoplasmatic reticulum and therefore this interaction might be important to keep PHD2 in the cytosol to regulate its function (30). Consistent with this assumption, FRET analysis and ITC measurements provide evidence for a tight interaction comparable to HIF-1α:HIF-1β interaction (28). However, a partial fraction of PHD2 was attached to ER and mitochondria. Additionally, PHD2 was found in the cytosol. Of note, neither PHD1 nor PHD3 contain a MYND-type Zn^{2+} finger domain suggesting a different protein regulation. N-terminal deletion of PHD2 renders PHD2 susceptible to degradation by Siah1/2 like PHD1 and PHD3 (38).

We identified the transmembrane domain of FKBP38 to be crucial for the interaction with PHD2. FKBP38 was described to be targeted to mitochondria and to ER and

moreover we observed binding of PHD2 to these organelles. Previously, mitochondrial pattern of PHD2 was already observed in rat tissues (39). The interaction domain containing the MYND-type Zn^{2+} finger domain of PHD2 in FKBP38 was suggested to be an anchor to organelles and thereby modulating PHD2 protein activity (40). We found that FKBP38 mediates the binding of PHD2 to ER and mitochondria and is required for PHD2 protein regulation. We explored the mechanism of PHD2 protein degradation. So far, we could not identify a specific protease or the ubiquitin-proteasome pathway to be involved in the degradation of PHD2. These data might suggest that a unique protease or a FKBP38-dependent degradation pathway is involved in the degradation of PHD2. Moreover, FKBP38 might be directly involved in regulation of PHD2 protein steady state levels by a so far unknown mechanism.

Interestingly, the PPIase Pin1 regulates the steady state levels of a variety of proteins to mediate proteasome-dependent and non-dependent proteasomal protein degradation (41), (42). Therefore, it might be most likely that PPIases may have additional functions to regulate the steady state levels of proteins. FKBP38 was also reported to have a chaperone activity monitored by *in vitro* refolding assays (43), (44). It remains open if this activity is involved in PHD2 regulation.

In summary, our results suggest that the transmembrane domain of FKBP38 is crucial for the FKBP38:PHD2 interaction and for the regulation of the PHD2 protein levels. Specific pharmacological disruption of FKBP38:PHD2 might be an interesting approach to modulate PHD2 protein abundance thereby influencing HIF-1α protein stability.

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Figure Legends

Figure 1. Detailed analysis of FKBP38 interaction domain.

(A) An array of 13mer peptides spanning the FKBP38 sequence was synthesized with forward-shifts by one amino acid. PHD2 interaction with the peptide array of the FKBP38 sequence forward (upper panel) and reverse (lower panel) was analyzed by Western blot. The respective binding motif comprised the peptides b13-24, which correspond to FKBP38 aa 37 to 56. The reverse sequence comprised of the same amino acids was not found to interact with PHD2 (lower panel). **(B)** Western blot analysis of the interaction between different PHD2 and a biotin-labeled FKBP38_37-56 peptide bound streptavidin matrix using anti-PHD2 antibodies. The streptavidin matrix alone served as control. **(C)** Co-IP analysis of endogenous proteins from rat liver lysate interacting with a FKBP38_37-56 affinity matrix using anti-PHD2 antibodies. The streptavidin matrix served as binding control.

Figure 2. Biochemical characterization of the FKBP38:PHD2 interaction.

(A) Fluorescence measurements at an excitation wavelength of 278 nm with 1 µM FKBP38 (__), 1 µM PHD2 (…) and a 1:1 mixture of both proteins (---). In addition, the calculated spectrum (__ __) represents the sum of the individual protein spectra, as it should appear when the components do not interact. **(B)** Fluorescence spectra of 1 µM FKBP38 (__), 1.25 µM PHD2_170-426 (…) and a 1:1 mixture of both proteins (---) at an excitation wavelength of 278 nm. In addition, the calculated spectrum (__ __) represents the sum of the individual protein spectra, as it should appear when the fluorescence of the proteins is not affected by the addition of a potential interaction partner. **(C)** Titration curve resulting from fluorescence measurements at 340 nm (excitation at 278 nm) of a sample containing 1 µM FKBP38/PHD2 and various concentrations of a peptide corresponding to

FKBP38_37-56. The straight line represents the fit according to the equation in the Materials and Methods section.

Figure 3. Mapping of the PHD2 interaction domain.

(A) Schematic representation of the PHD2 domain architecture and the used PHD2 constructs. (**B**) IVTT ^{35}S-labeled PHD2 variants were allowed to bind to GST-FKBP38 or GST alone; protein complexes were pulled-down by glutathione sepharose, separated by SDS-PAGE and visualized by phosphorimaging. **(C and D)** IVTT ^{35}S-labeled FKBP38 was allowed to interact with GST-PHD2_1-426, GST-PHD2_170-426, GST-PHD2_1-31, GST-PHD2_1-58, GST-PHD2_1-114 or GST alone. GST pull-down was done described above.

Figure 4. FKBP38 regulates PHD2 protein abundance.

(A) Cellular extracts from FKBP38 wild-type or knockout MEFs incubated for 8 h at 20% O_2 or 0.2% O_2 were extracted with RIPA buffer, separated by SDS-PAGE and endogenous FKBP38, PHD2 and β-actin were detected by immunoblotting. **(B)** Total RNA was extracted from *Fkbp38+/+* and *Fkbp38-/-* MEFS cultivated at normoxia and mRNA levels of mPHD1, mPHD2 and mPHD3 and mS12 were quantified by real-time RT-qPCR. Transcript levels of these genes were normalized to mS12 (mean of n = 2).

Figure 5. Proteolytic regulation of PHD2.

Cellular extracts were prepared, separated by SDS-PAGE and analyzed by Western blotting. **(A)** MCF-7 cells were incubated at 20% O_2 with indicated protease inhibitors: E64 (50 μM), AEBSF (300 μM), MG132 (10 μM), ALLM (10 μM), Pepstatin (10 μM). DMSO and EtOH served as solvent control. **(B)** MCF-7 cells were pre-incubated for 24 h at 0.2% O_2 and then re-oxygenated in the presence of indicated protease inhibitors described under A. **(C)** Mouse ts20 cell line was incubated either under 34ºC or 39ºC for 24, 48, 72 h and cellular extracts were analyzed by immunoblotting. **(D)** Mouse ts20 cell line reconstituted with a wild-type E1 gene (H38-5) were incubated under the same conditions described in (C) and cellular proteins were analyzed by Western blotting**. (E)** HeLa cells were treated with 100 μM CHX and incubated for 24, 48, 72 h at 20% O_2 or 0.2% O_2. Cellular extracts were analyzed by immunoblotting. **(F)** HeLa cells were treated with 100 μM CHX and

incubated for 24, 48, 72 h at 20% O_2 or 0.2% O_2. PHD2 protein levels were quantified by densitometry and results are mean values of relative intensity normalized to time point 0 ± SEM of three independent experiments.

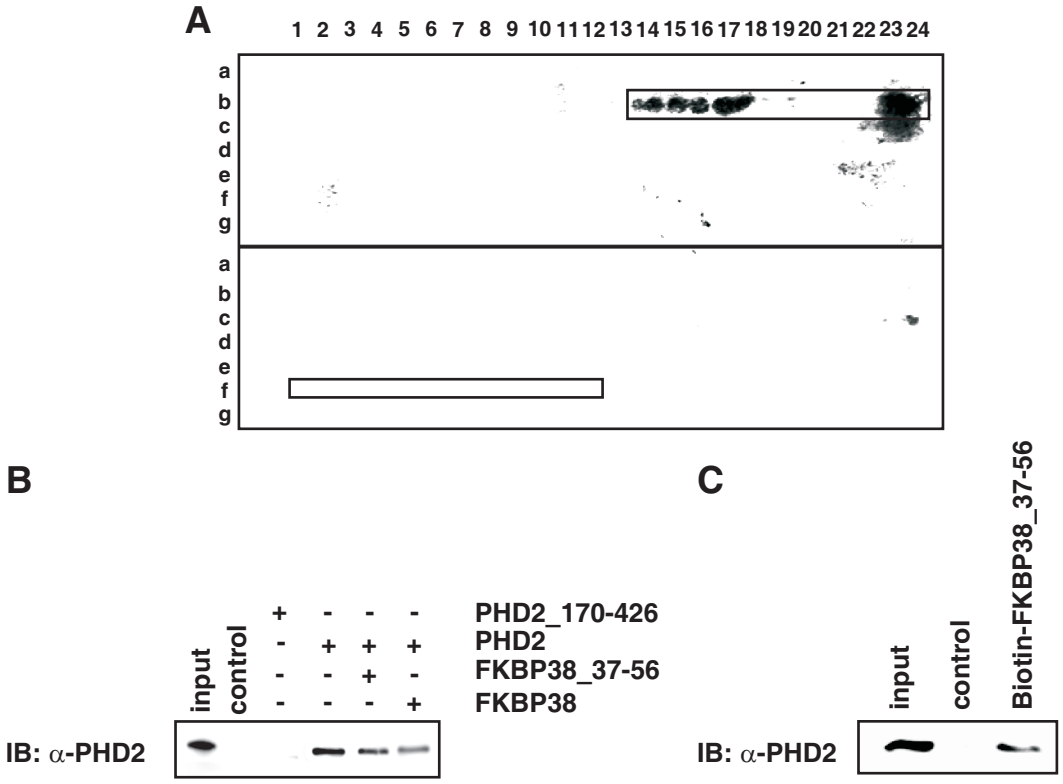
Figure 6. FKBP38 transmembrane domain determines PHD2 protein stability.
(A-C) HeLa cells were transiently transfected with pcDNA3.1-nV5-FKBP38, FKBP38Δ98-257, FKBP38_1-389. After fixation, cells were stained with V5, DAPI and either with mitotracker (mitochondria marker), calreticulin (ER marker) or with WGA (golgi marker). **(D)** MCF-7 cells were transiently transfected with Gal4-DBD_HIF-1α-370-429_VP16-AD expression vector (schematically represented), Gal4 response element-driven firefly luciferase reporter, renilla luciferase control vector and either co-transfected with V5-FKBP38, V5-FKBP38Δ98-357, V5-FKBP38_1-389 or mock (pcDNA3.1-nV5-LacZ) transfected. 24 h post-transfection, cells were either cultured under normoxic or hypoxic conditions for additional 16 h and firefly luciferase activities were determined and corrected for renilla luciferase activity. Results are mean values of relative luciferase activities ± SEM of 4 independent experiments performed in triplicates. *P* values were obtained by paired t tests (** $p<0.01$; * $p<0.05$). Expression of the transfected V5-tagged vectors was verified by immunoblotting against V5 and β-actin. **(E)** HeLa FKBP38 RNAi control cells (ctr, 3A5) and FKBP38 RNAi depleted cells (3D6, 2G8) were transiently transfected with HIF-1α one-hybrid construct, Gal4 response element-driven firefly luciferase reporter, renilla luciferase control vector and with indicated FKBP38 expression plasmids. 24 h post-transfection, cells were cultivated for 16 h under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions before relative luciferase activities were determined. Results are mean values ± SEM of 7 independent experiments performed in triplicates. **(F)** HeLa wild-type, control or FKBP38-depleted cells (3D6, 2G8) were transiently transfected with indicated plasmids and PHD2, V5 and β-actin were detected by immunoblotting. **(G)** HeLa wild-type, control or FKBP38 depleted cells (3D6, 2G8) were transiently transfected with indicated plasmids and hydroxylation activity was measured using a VBC-binding assay. Shown are mean values of relative VBC binding ± SEM of 2 independent experiments performed in triplicates.

Figure 7. Requirement of FKBP38 localization for PHD2 interaction.

(A) HEK293 cells were transiently transfected with indicated ECFP and EYFP vectors and FRET was monitored. **(B)** Recombinant GST, GST-FKBP38 or GST-FKBP38_1-389 were incubated with radioactive labeled ^{35}S-PHD2 and protein complexes were pulled-down by glutathione sepharose, separated by SDS-PAGE and visualized by phosphorimaging. **(C)** HeLa cells were transiently transfected with Gal4-DBD and VP16-AD fusion protein vectors, Gal4 response element-driven firefly luciferase reporter as well as a renilla luciferase control vector. Following transfection, the cells were incubated under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions and luciferase reporter gene activities were determined 16 h later. Firefly to renilla luciferase activity ratios were normalized to the normoxic negative control DBD-p53/AD-CP co-transfection which was arbitrarily defined as 1. Mean values ± SEM are shown of 3 independent experiments performed in triplicates.

Figure 8. Sub-cellular localization of FKBP38 and PHD2.

HeLa wild-type cells or FKBP38-silenced cells (2G8) were incubated at 20% O_2 **(A), (B)** or at 0.2% O_2 **(C), (D)** and cellular membranes were separated from cytosolic fractions by differential centrifugation and then separated in a 10% to 30% iodixanol (Opti-prep) gradient. 1 ml fractions were collected and analyzed by immunoblotting. Mitofilin served as mitochondria and protein disulfide isomerase (PDI) as endoplasmatic reticulum (ER) marker. **(E)** Cytosolic fractions of HeLa wt and 2G8 incubated at 20% O_2 or 0.2% O_2 were analyzed by immunoblotting.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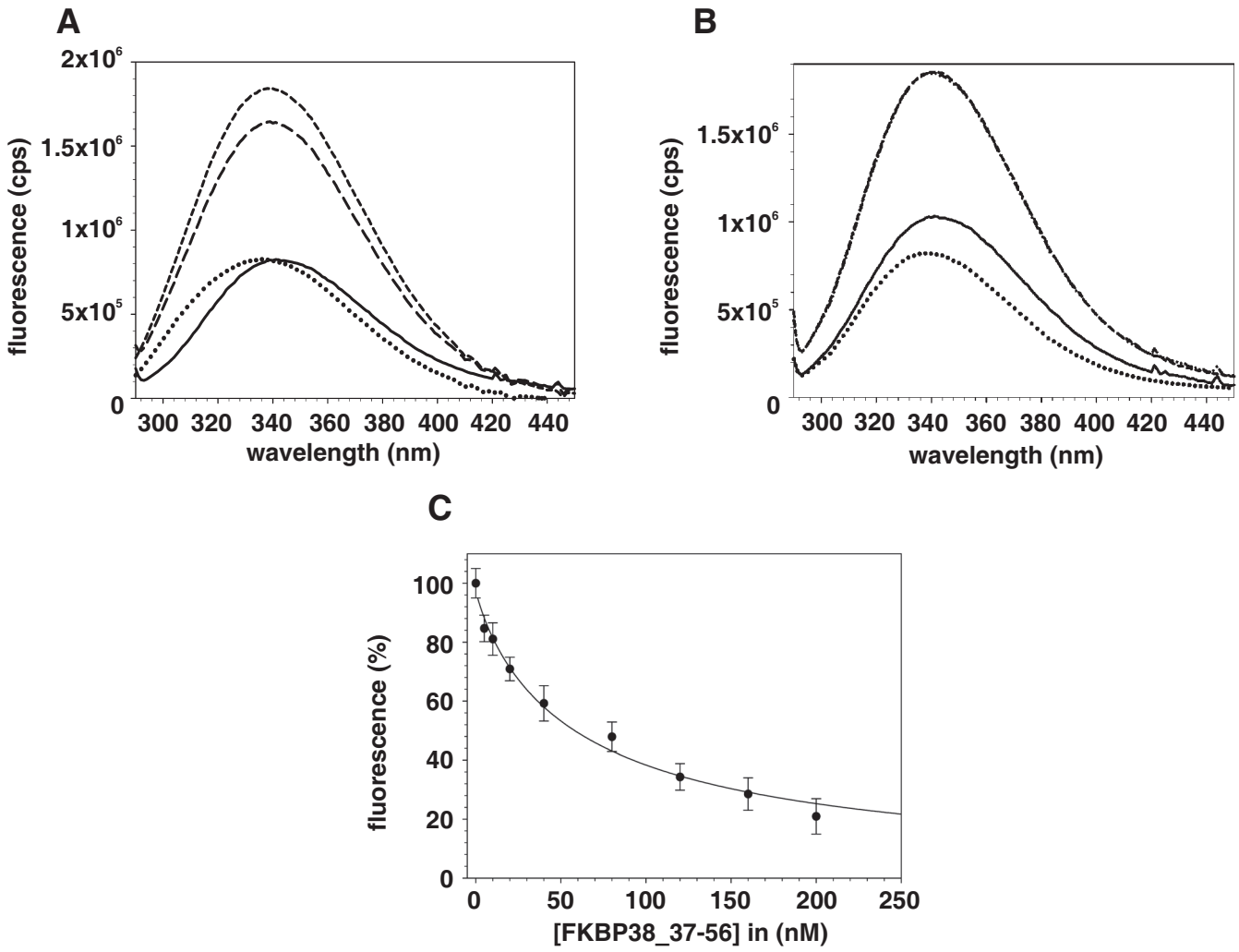
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
a b c d e f g
a b c d e f g
B
input
control
+ - - - PHD2_170-426
- + + + PHD2
- - + - FKBP38_37-56
- - - + FKBP38
IB: α-PHD2
C
input
control
Biotin-FKBP38_37-56
IB: α-PHD2

Figure 1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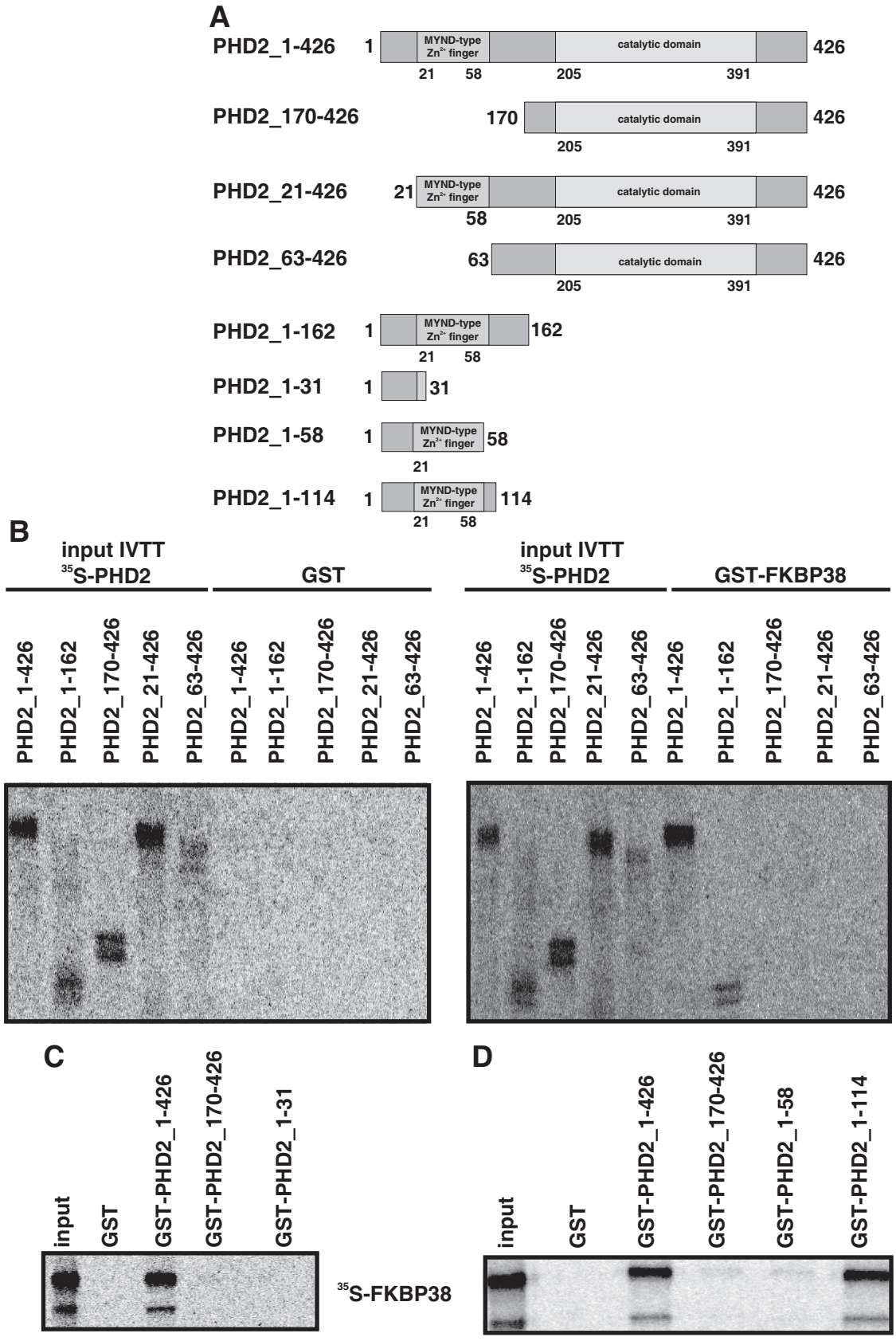
2x10^6
1.5x10^6
1x10^6
5x10^5
0
fluorescence (cps)
300 320 340 360 380 400 420 440
wavelength (nm)
B
1.5x10^6
1x10^6
5x10^5
0
fluorescence (cps)
300 320 340 360 380 400 420 440
wavelength (nm)
C
100
80
60
40
20
0
fluorescence (%)
0 50 100 150 200 250
[FKBP38_37-56] in (nM)

Figure 2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MYND-type Zn2+ finger
catalytic domain
426
21
58
205
391
PHD2_170-426
170
catalytic domain
426
205
391
PHD2_21-426
21
MYND-type Zn2+ finger
catalytic domain
426
58
205
391
PHD2_63-426
63
catalytic domain
426
205
391
PHD2_1-162
1
MYND-type Zn2+ finger
162
21
58
PHD2_1-31
1
31
PHD2_1-58
1
MYND-type Zn2+ finger
58
21
PHD2_1-114
1
MYND-type Zn2+ finger
114
21
58
B
input IVTT 35S-PHD2
GST
input IVTT 35S-PHD2
GST-FKBP38
PHD2_1-426
PHD2_1-162
PHD2_170-426
PHD2_21-426
PHD2_63-426
C
input
GST
GST-PHD2_1-426
GST-PHD2_170-426
GST-PHD2_1-31
35S-FKBP38
D
input
GST
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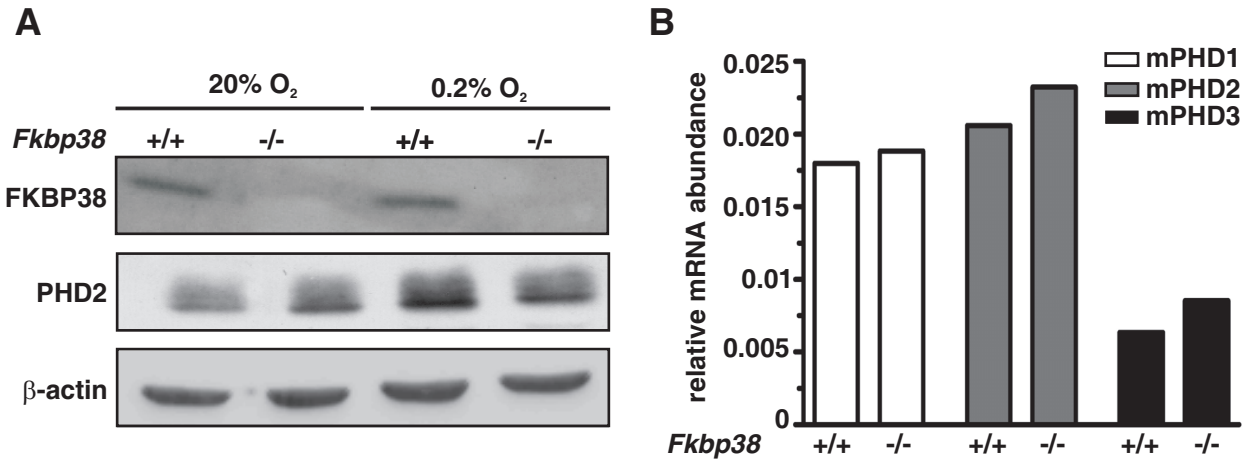
GST-PHD2_170-426
GST-PHD2_1-58
GST-PHD2_1-114

Figure 3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20% O2
0.2% O2
Fkbp38
+/+
-/-
+/+
-/-
FKBP38
PHD2
β-actin
B
mPHD1
mPHD2
mPHD3
relative mRNA abundance
0.025
0.020
0.015
0.01
0.005
0
Fkbp38
+/+
-/-
+/+
-/-
+/+
-/-

Figure 4

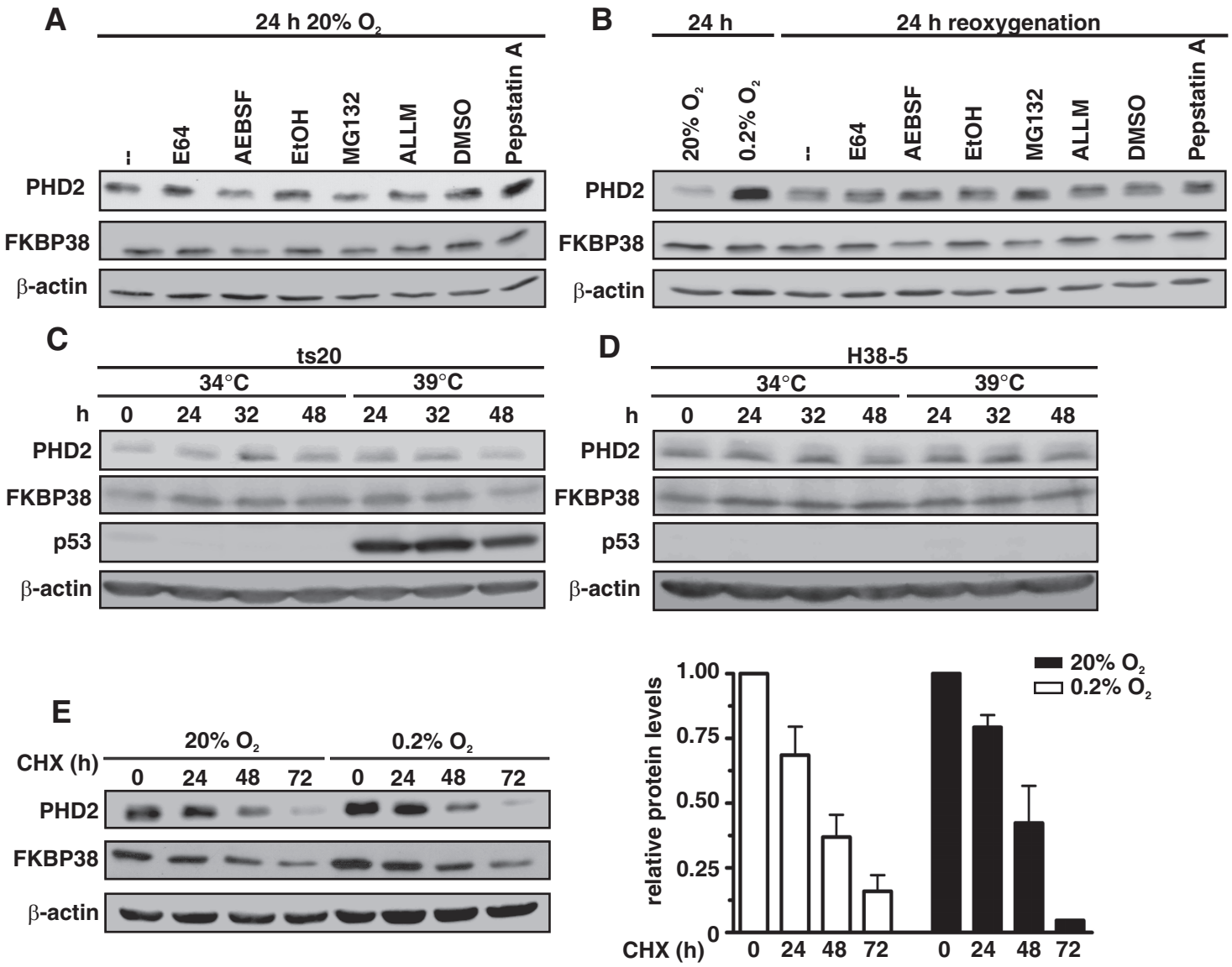
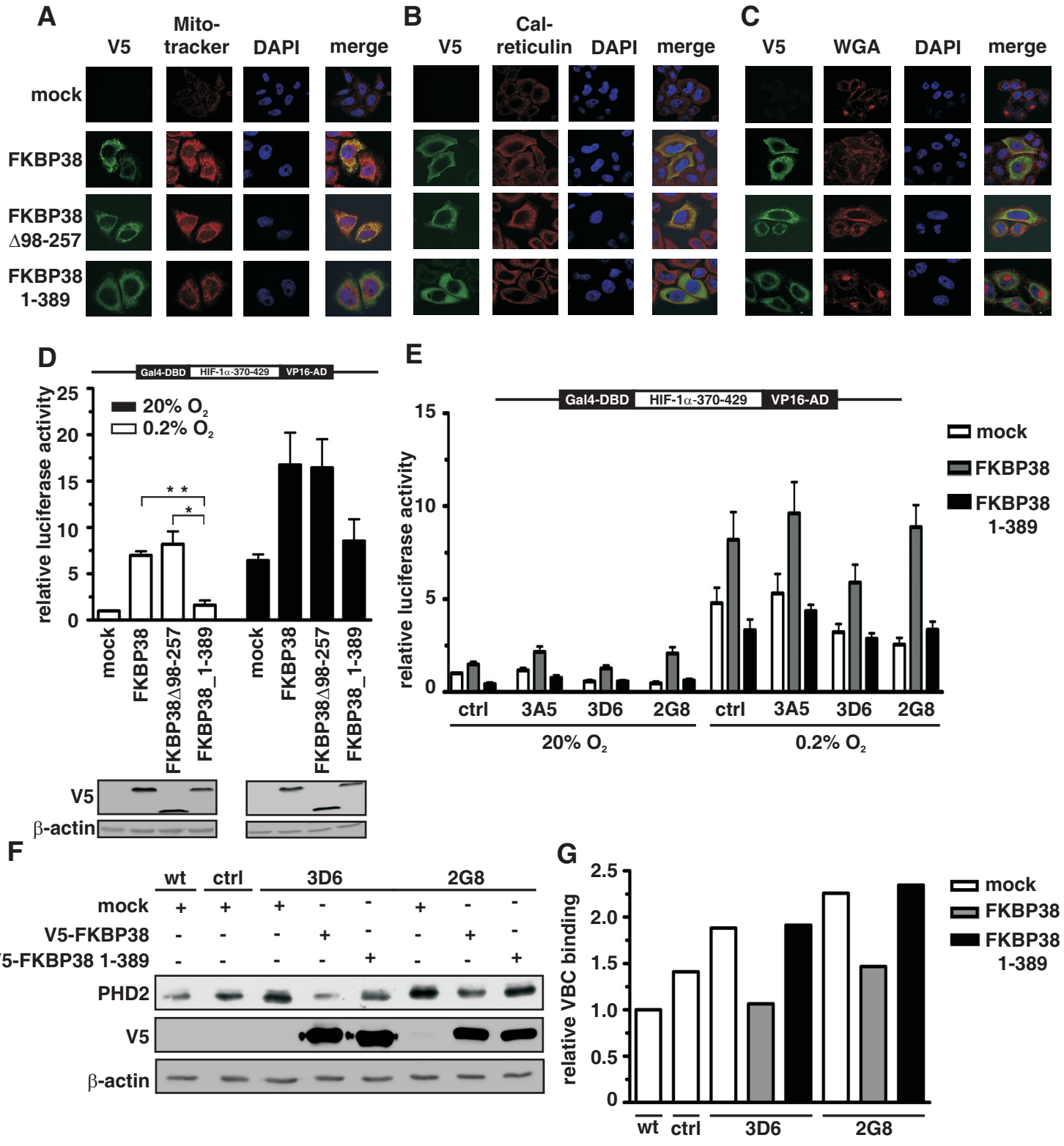

A
24 h 20% O2
--
E64
AEBSF
EtOH
MG132
ALLM
DMSO
Pepstatin A
PHD2
FKBP38
β-actin
B
24 h
24 h reoxygenation
20% O2
0.2% O2
--
E64
AEBSF
EtOH
MG132
ALLM
DMSO
Pepstatin A
PHD2
FKBP38
β-actin
C
ts20
34°C
39°C
h
0
24
32
48
24
32
48
PHD2
FKBP38
p53
β-actin
D
H38-5
34°C
39°C
h
0
24
32
48
24
32
48
PHD2
FKBP38
p53
β-actin
E
20% O2
0.2% O2
CHX (h)
0
24
48
72
0
24
48
72
PHD2
FKBP38
β-actin
20% O2
0.2% O2
relative protein levels
1.00
0.75
0.50
0.25
0
CHX (h)
0
24
48
72
0
24
48
72

Figure 5


A
V5
Mito-tracker
DAPI
merge
mock
FKBP38
FKBP38 Δ98-257
FKBP38 1-389
B
V5
Cal-reticulin
DAPI
merge
C
V5
WGA
DAPI
merge
D
Gal4-DBD
HIF-1α-370-429
VP16-AD
relative luciferase activity
20% O2
0.2% O2
mock
FKBP38
FKBP38Δ98-257
FKBP38_1-389
V5
β-actin
E
Gal4-DBD
HIF-1α-370-429
VP16-AD
relative luciferase activity
mock
FKBP38
FKBP38 1-389
ctrl
3A5
3D6
2G8
20% O2
0.2% O2
F
wt
ctrl
3D6
2G8
mock
V5-FKBP38
V5-FKBP38 1-389
PHD2
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β-actin
G
relative VBC binding
mock
FKBP38
FKBP38 1-389
wt
ctrl
3D6
2G8

Figure 6

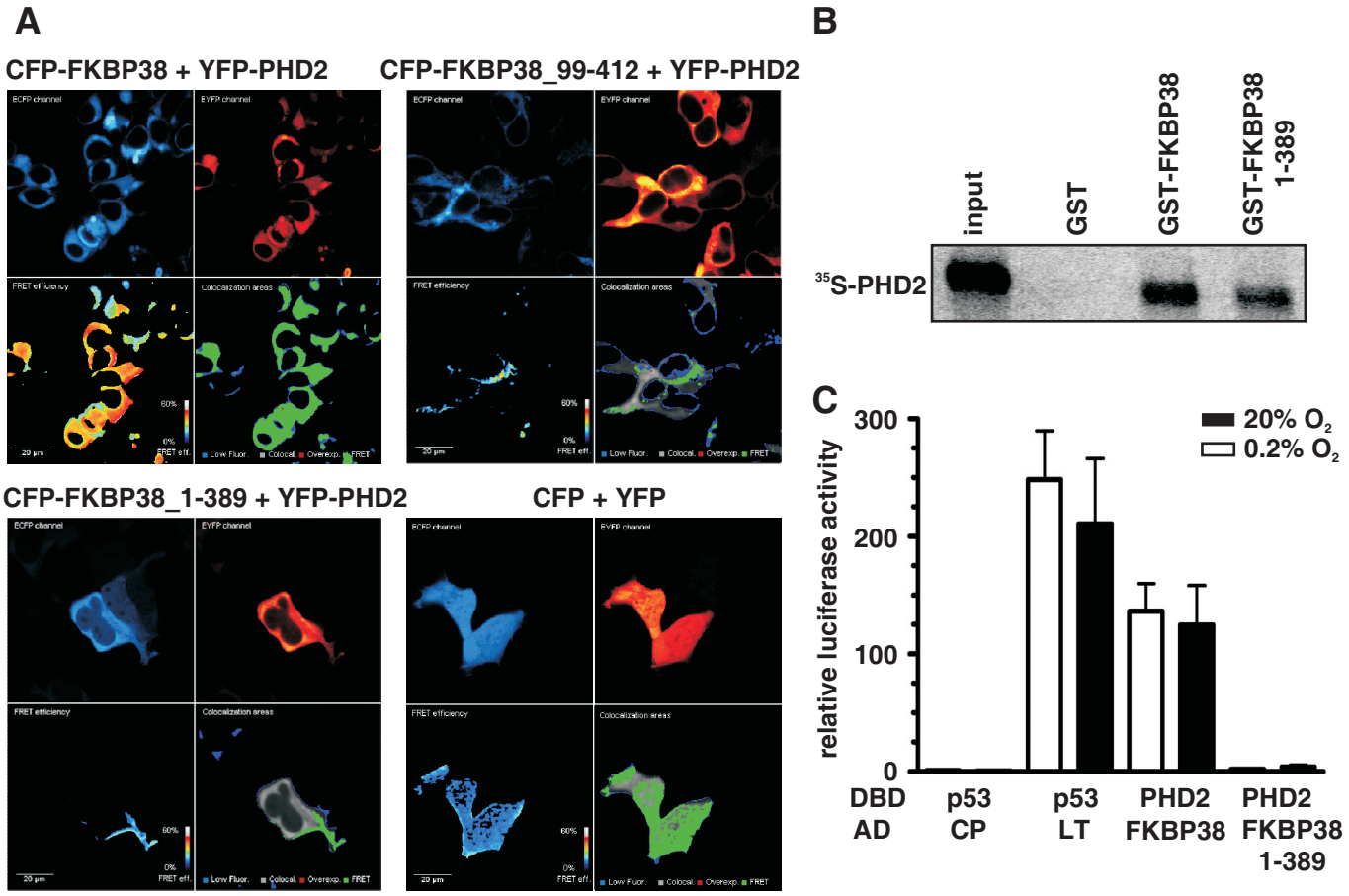
A
CFP-FKBP38 + YFP-PHD2
CFP-FKBP38_99-412 + YFP-PHD2
CFP-FKBP38_1-389 + YFP-PHD2
CFP + YFP
ECFP channel
EYFP channel
FRET efficiency
Colocalization areas
B
input
GST
GST-FKBP38
GST-FKBP38 1-389
35S-PHD2
C
relative luciferase activity
300
200
100
0
20% O2
0.2% O2
DBD AD
p53 CP
p53 LT
PHD2 FKBP38
PHD2 FKBP38 1-389

Figure 7

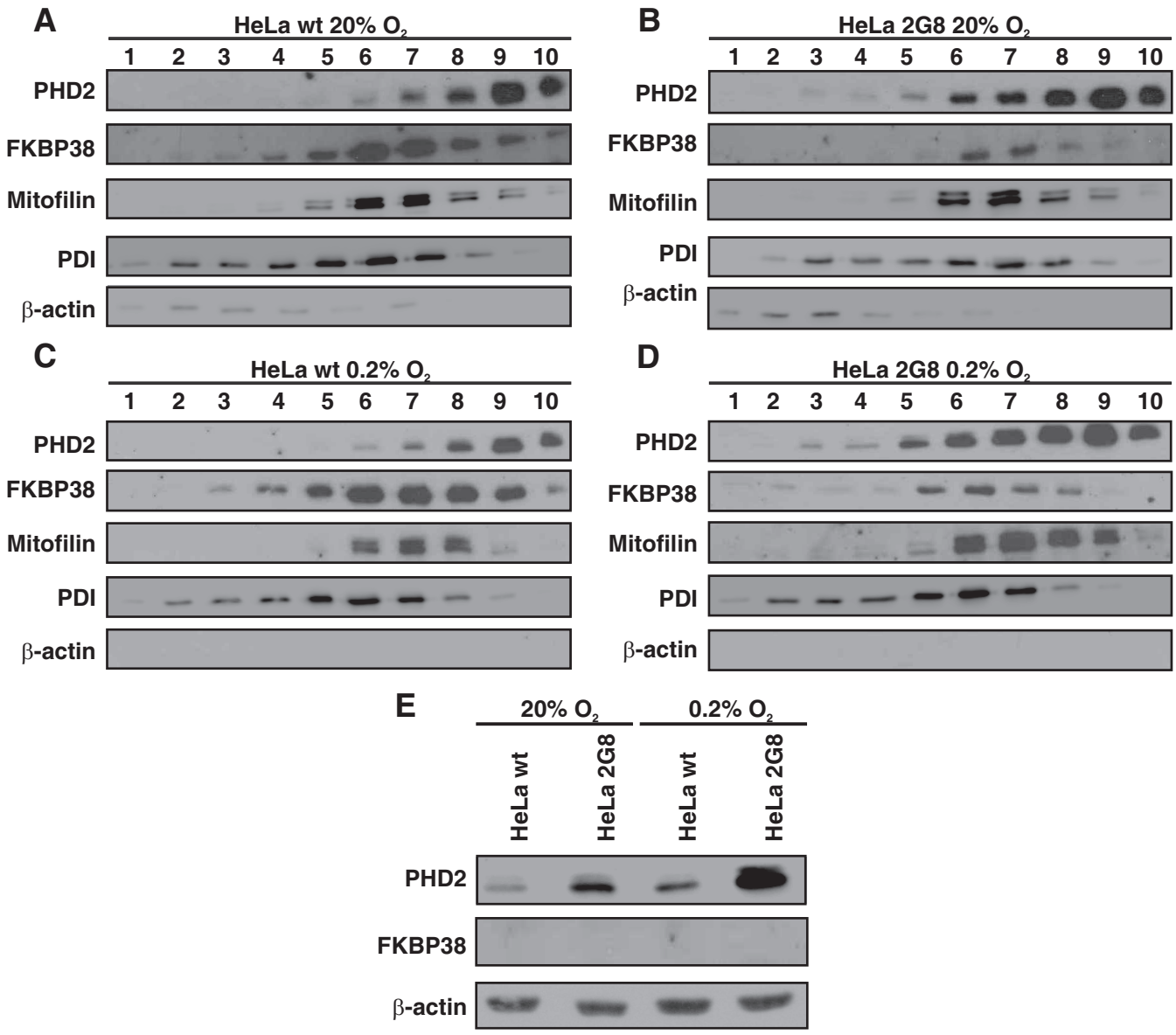
A
HeLa wt 20% O2
1 2 3 4 5 6 7 8 9 10
PHD2
FKBP38
Mitofilin
PDI
β-actin
B
HeLa 2G8 20% O2
1 2 3 4 5 6 7 8 9 10
PHD2
FKBP38
Mitofilin
PDI
β-actin
C
HeLa wt 0.2% O2
1 2 3 4 5 6 7 8 9 10
PHD2
FKBP38
Mitofilin
PDI
β-actin
D
HeLa 2G8 0.2% O2
1 2 3 4 5 6 7 8 9 10
PHD2
FKBP38
Mitofilin
PDI
β-actin
E
20% O2
0.2% O2
HeLa wt
HeLa 2G8
HeLa wt
HeLa 2G8
PHD2
FKBP38
β-actin

Figure 8

7 List and contributions to the publications and manuscript

This thesis consists of the following original publications and manuscript and I contributed to it as indicated:

Publications:

Barth S., Nesper J., Hasgall P.A., Wirthner R., Nytko K.J., Edlich F., Katschinski D.M., Stiehl D.P., Wenger R.H. and Camenisch G. (2007) The peptidyl prolyl cis/trans isomerase determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. **Mol Cell Biol**; 27(10):3758-68

- Everything, except yeast-two hybrid screening, figure 1A-C, figure 2 and figure 5A

Wirthner R., Kuppusamy B., Stiehl D.P., ***Barth S.***, Spielmann P., Oehme F., Flamme I., Katschinski D.M., Wenger R.H., Camenisch G. (2007) Determination and modulation of prolyl-4-hydroxylase domain (PHD) oxygen sensor activity. **Methods Enzymol**; 435:43-60

- Establishment of VBC-dependent hydroxylation assay for cell extracts
- Figure 3.2 (D)

Manuscript:

Barth S., Edlich F., Berchner-Pfannschmidt U., Gneuss S., Shirane M., Wenger R.H. and Camenisch G. HIF prolyl-4-hydroxylase PHD2 protein stability depends on FKBP38 sub-cellular localization.

- Everything except figure 1, 2 and FRET analysis (Fig. 7A)

8 Unpublished data

Functional relevance of the FKBP38:PHD2 interaction in cell growth, proliferation and cell death

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Introduction

Oxygen is an essential regulator of cellular metabolism and cell survival or death. Adaptation to oxygen deprivation is mainly governed by the transcription factor HIF and the stability of HIF-α subunits is oxygen-dependently regulated by prolyl-4-hydroxylases (PHDs). PHDs require oxygen as a substrate and therefore HIF-α degradation is a function of oxygen availability. In addition to the function of PHDs as cellular oxygen sensors, the functional homologue of PHD in *Drosophila* (Hph) has been implicated in the regulation of cell growth (cell size), suggesting a link between growth signals, mitochondrial activity and oxygen sensing (1). *Drosophila* cyclin-dependent protein kinase complex cyclin D/Cdk4 requires Hph to promote cell growth but not for cell proliferation. Adaptive mechanisms to O_2 deprivation include inhibition of energy-consuming mRNA translation and it has been shown that hypoxia inhibits multiple key regulators of the PI3K/TSC/mTOR pathway (2). Interestingly, cell size regulation has been shown to depend also on FKBP38 (3). Overexpression of TSC1 resulted in upregulation of FKBP38 expression and reduced cell size by down-regulating mTOR activity, and this effect was reverted by downregulation of FKBP38 by anti-sense oligonucleotides.

In addition, FKBP38 has been linked to cell death. An interaction of FKBP38 with Bcl-2 has been described, but the functional consequence of this association remains controversial. Pro- as well as anti-apoptotic effects have been reported depending on the used cell model (4), (5). Shirane et al. reported that in HeLa cells FKBP38 recruits Bcl-2 to mitochondria and promotes cell survival. In SH-SY5Y neuroblastoma cells down-regulation of FKBP38 by RNAi reduced cell death after induction of apoptosis with etoposide, staurosporine and camptothecin (4). Interestingly, a function of PHDs in apoptosis is supported by the observation that *Egl9-/-* (*C. elegans* PHD homolog) worms are resistant to certain neurotoxins (6). A pro-apoptotic function of human PHD3 has been shown in adrenal medullary tumors or

after nerve growth factor withdrawal (7), (8). Similar to PHD3, the rat homolog SM-20 which is located to mitochondria is a mediator of caspase-dependent cell death after NGF deprivation (9). Interestingly, forced expression of PHD3 promotes aggregation of proteasomal components leading to cell death (10).

Hence, we wanted to explore the role of FKBP38 in cell death and analyze FKBP38-dependent regulation of PHD2 protein levels in relation to cell growth, proliferation and death or survival.

Methods and materials

Cell lines and cell culture

Wild-type and stable control RNAi or FKBP38 RNAi transfected human HeLa cervical carcinoma cells were cultured in high-glucose Dulbecco`s modified Eagle`s medium (DMEM) (Sigma) supplemented with 10% FCS (Gibco) and Penicillin/Streptomycin (Sigma) and incubated at 20% O_2, 5% CO_2, 37ºC. For serum starvation experiments, cells were cultured with DMEM and 0.1% FCS for indicated periods of time.

Western Blot analysis

Total cell extracts of cultured cells were prepared using RIPA buffer containing 50 mM Tris Cl pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS. Protein concentration was determined by detergent-insensitive BCA assay (Pierce, Perbio Science, Lausanne, Switzerland) using bovine serum albumin as a standard. Immunoblot analysis were performed as described previously (11). Briefly, proteins were separated by SDS-PAGE, electro-transferred onto nitrocellulose membranes (Amersham Biosciences) and incubated with anti-PHD2 (Novus, Abcam, Camridge. United Kingdom), anti-FKBP38 (4), anti-phospho-S6-kinase (Cell signaling technology, Bioconcept, Allschwil, Switzerland) and anti-S6 kinase (Cell signaling technology, Bioconcept, Allschwil, Switzerland) antibodies.

Cell analysis and 5-bromo-2-deoxyuridine (BrdU) proliferation assay

HeLa cells were seeded in cell culture dishes and counted at different time points. Cell number, viability and size were determined using a Vi-Cell cell counter (Beckman Coulter, Switzerland). To measure the incorporation of BrdU in place of thymidine into the DNA, the cell proliferation BrdU assay from Roche was used according to the manufactures instructions. Briefly, HeLa cells were seeded at a concentration of 3000 cells/well in a 96 well luminometer plate. The cells were incubated with 10 μM BrdU for 2 h. To detect the incorporated BrdU, the fixed cells

were incubated with anti-BrdU-POD antibody and the chemiluminescence was monitored by a Centro LB 960 Luminometer (Berthold, Regensdorf, Switzerland).

Caspase 3 activity assay with DEVD-AMC fluorogenic substrate

HeLa cells were exposed to 44ºC for 4 h and further incubated at 37ºC for 4 h. Cells were pelleted after washing by centrifugation and resuspended in lysis buffer containing 100 mM HEPES, 0.1% CHAPS, 10% sucrose, 1 mM EDTA, 1 mM phenylmethylsuflonyl fluoride (PMSF). 60 μg of cellular proteins were incubated with 3 mM of the fluorogenic substrate DEVD-AMC (Biomol) in a 96-well microtiter plate. The cleavage of the DEVD-AMC was immediately monitored by the release of AMC in a fluoroscan plate reader (Tecan) using 360 nm for excitation and 465 nm for emission. Fluorescence was measured for 18 min.

Results and discussion

Loss of function mutations of *Drosophila* prolyl-4-hydroxylase Hph suppresses cyclin D/Cdk4 induced cell growth but not cell proliferation suggesting that Hph is a positive regulator of cell growth (1). Cell growth is mainly controlled by the activity of the mTOR pathway. Recently, FKBP38 has been reported to limit the activity of mTOR by binding directly to mTOR. FKBP38:mTOR interaction is competed by Rheb-GTP binding to FKBP38. Rheb-GTP accumulates under growth supporting conditions whereas under growth factor withdrawal or nutrient deprivation Rheb-GDP can be found (12). We hypothesized that in our HeLa FKBP38 RNAi cell model, we will observe an increase in cell size as a result of increased PHD2 protein levels and increased mTOR target phosphorylation due to loss of FKBP38.

Hence, we monitored the cell size of the FKBP38-downregulated cells 3D6 and 2G8 compared to the parental (HeLa wt) and control (HeLa ctrl) cells (previously described (11)). We did not observe a significant change in the cell size which was dependent on the loss of FKBP38 (Fig. 1A). The same results were observed by FACS analysis (data not shown).

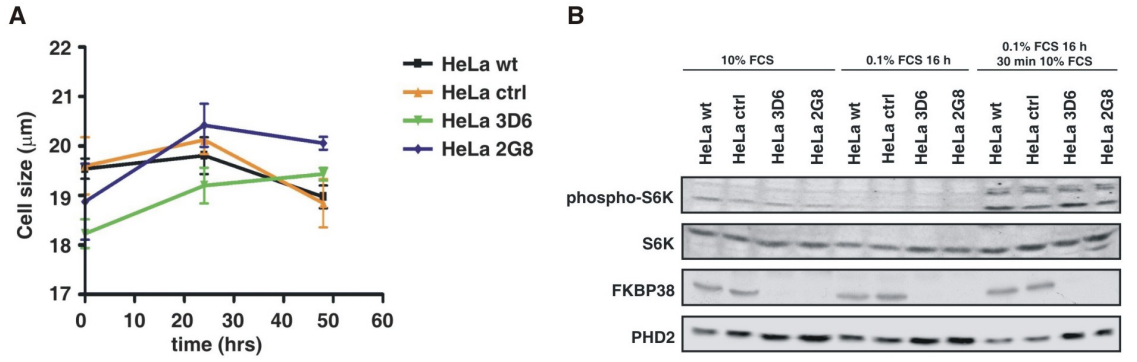

Figure 1. Depletion of FKBP38 does not significantly influence mTOR activity.

(A) HeLa cell lines were cultured for the indicated time and the cell size was determined by Via-Cell counter. Shown are mean values ± SEM of 4 independent experiments. (B) HeLa cells were incubated for 16 h either with 10% FCS or 0.1% FCS and afterwards re-stimulated with 10% FCS. Cellular extracts were prepared and PHD2, FKBP38, phospho-SK6 and S6K were detected by immunoblotting. Total S6K protein levels served as loading control.

Furthermore, HeLa cells were incubated with 10% fetal calf serum (FCS) or 0.1% FCS for 16 h. Additionally, HeLa cells were serum starved with 0.1% FCS and re-stimulated with 10% FCS for 30 min. Cellular extracts were prepared and FKBP38, PHD2, phospho-SK6 and total S6K were detected. We observed an increase of PHD2 protein abundance in the FKBP38-depleted cells which was not affected by serum starvation and re-stimulation with 10% FCS (Fig. 1B). FKBP38 protein levels were not affected. Because of the proposed inhibitory function of FKBP38 on the mTOR activity, we expected to see an increase in phospho-S6K in the FKBP38 depleted cells because S6K is a known phosphorylation target of mTOR. However, in our hands we did not detected differences in the phosphorylation state of S6K in dependency of FKBP38. We observed a general increase in the phosphorylation state of S6K after serum starvation and re-stimulation with FCS.

Next, we examined the cell proliferation of wild-type HeLa cells compared to stably control transfected HeLa cells (HeLa ctrl) and stably transfected HeLa cells with a FKBP38 RNAi expressing plasmid (HeLa 3D6, 2G8).

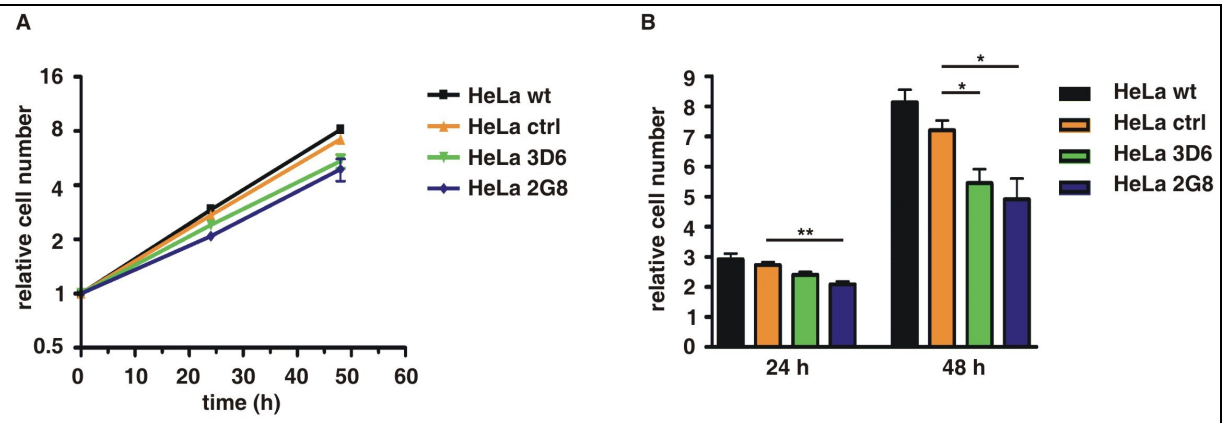

Figure 2. The proliferation is decreased in HeLa FKBP38-silenced cells.

HeLa cells were seeded with a cell density of 300'000/10 cm dish. Cells were counted after 24 h which was defined as time point t0. Cells were grown for further 24, 48 h and the number of cells were counted. Results are mean values of relative cell numbers normalized to t0 ± SEM of 4 independent experiments. *P* values were obtained by paired t tests (** p<0.01; * p<0.05).

Interestingly, we observed a significant slower proliferation of the FKBP38 RNAi cells 3D6, 2G8 after 24 h and 48 h compared to wild-type HeLa cells and HeLa ctrl cells (Fig. 2). The HeLa ctrl cell line proliferated slightly slower compared to the HeLa wild-type cells which is most likely due to the incubation of the HeLa ctrl, 3D6, 2G8 with hygromycin B containing DMEM medium. In hypoxia, the proliferation rates did not significantly differ between the FKBP38 RNAi cells 3D6, 2G8 and the control cell lines HeLa wt and ctrl after 24 h (Fig. 3). However, when we tried to determine the changes in the DNA synthesis rate in these cells by the 5-bromo-2-deoxyuridine (BrdU) assay we did not see a significant difference in the uptake of BrdU between the ctrl and the FKBP38-silenced cell lines (Fig. 4). A slight difference was observed between the parental cell line and the ctrl and FKBP38 silenced cells which was most likely due to incubation of the cells with the antibiotic hygromycin B which functions as a selection marker. To confirm these changes in cell proliferation FKBP38 reconstitution or PHD2 RNAi experiments should be done to investigate the dependency of cell proliferation on FKBP38 and/or PHD2.

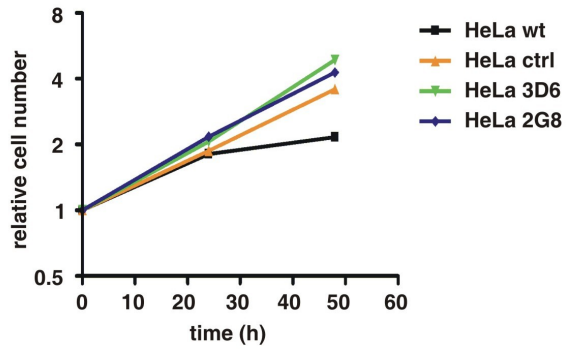

Figure 3. The influence of hypoxia on the proliferation rate on HeLa wt, ctrl and FKBP38-depleted cells.

HeLa cells were seeded at a cell density of 300'000 cells/10 cm dish and incubated under 0.2% O_2. Cells were counted after 24 h which was defined as time point t0. Cells were grown for further 24, 48 h and the number of cells was counted. Results are mean values of one experiment.

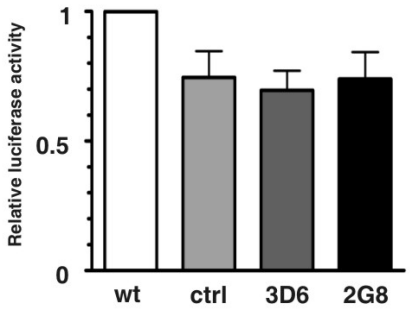

Figure 4. DNA synthesis rate was not affected by loss of FKBP38.

HeLa cells were seeded at a cell density of 300'000 cells/10 cm dish and incubated under 0.2% O_2. Cells were counted after 24 h which was defined as time point t0. Cells were grown for further 24, 48 h and the number of cells was counted. Results are mean values of relative cell luciferase counts normalized HeLa wt ± SEM of 4 independent experiments.

Because the variations in cell proliferation were not caused by alterations in the DNA synthesis, we analyzed the influence of FKBP38 on cell viability and cell death.

Hence, we monitored the number of dead cells by trypan blue staining in relation to total counted cells. In FKBP38 down-regulated cells the cell viability tended to be lower in the FKBP38-silenced cells (Fig. 5A). The decrease in cell viability might explain the lower cell number counted in the FKBP38 RNAi cells.

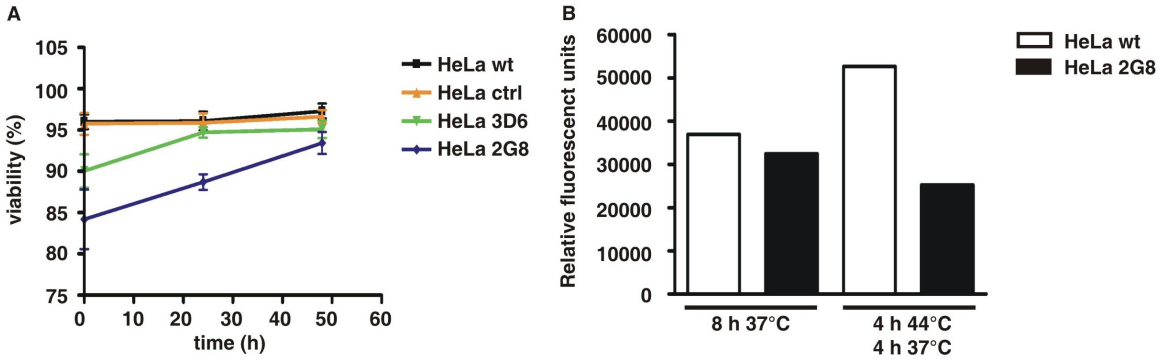

Figure 5. Analysis of cell death by trypan blue staining and apoptosis by measurement of caspase-3 activity in FKBP38-down-regulated cells.

(A) Cell viability was monitored by trypan blue exclusion and the number of viable cells was normalized to total number of cells (in %). Shown are mean values ± SEM of 4 independent experiments. (B) HeLa cells were cultivated for the indicated time periods at 37°C or 44°C and caspase-3 activity of total cellular lysates was measured.

Cells may die by an induced intracellular program (apoptosis) after death stimuli or by necrosis caused from acute tissue injury which provokes an inflammory response. Inhibition of FKBP38 reduces etoposide-induced apoptosis in neuroblastoma cells and has neuroprotective effects in a rat brain ischemia model, suggesting a pro-

apoptotic function of FKBP38 (4), (13). To investigate a possible role of PHD2 and FKBP38 in apoptosis, we cultivated HeLa wild-type and FKBP38-silenced HeLa 2G8 cells under 37°C or 44°C. Whereas no significant difference was observed at 37°C, 2G8 cells showed reduced apoptosis compared to wild-type cells after heat shock treatment (Fig. 5B).

Our initial data indicate a reduced caspase-3 activity after heat-induced apoptosis in our stable FKBP38 siRNA HeLa cell clones, supporting a pro-apoptotic function for FKBP38. Contrary, cell viability was decreased at the early time points. It might be as well that the cells were more prone to stress resulting in increased necrotic cell death. However, this has to be confirmed by additional assays like staining for HMG-1 which is a marker for necrotic cell death.

To investigate the role of FKBP38 in apoptosis, similar experiments should be performed with alternative apoptosis inducers (e.g. etoposide, irradiation) and apoptosis assessed by measuring caspase-3 activity as well as by propidium iodide/annexin V staining and FACS analysis. To investigate the influence of hydroxylase activity, the experiment might be done under low-oxygen conditions or in the presence of PHD inhibitors. To investigate whether differences in apoptosis can be affected by PHD2 expression, we would transiently down-regulate PHD2 by siRNA in FKBP38-depleted clones and vice-versa overexpress PHD2 in wild-type HeLa cells, respectively. In addition, we would generate stable PHD2 down-regulated HeLa as well as HEK293 cell clones and apoptosis would be measured in these clones as mentioned above. Accordingly, to verify a direct function of FKBP38 in apoptosis, FKBP38 expression in siRNA clones will be reconstituted by transient transfections.

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9 Conclusions

Cells developed a variety of strategies to respond to diverse stress stimuli. Oxygen deprivation results in the stabilization of the HIF transcription factor that allows the expression of so far more then 70 proven target genes and more than 200 potential target genes involved in the regulation of cellular energy metabolism, cell growth and cell survival or cell death and cellular, local and systemic oxygen supply (1). However, the stability of the HIF-α subunits is tightly regulated by the oxygen-dependently regulated prolyl-4-hydroxylases PHDs. So far, at least three isoforms (PHD1-3) are known to modulate HIF-α subunits *in vitro* and PHD2 has been suggested to be the main oxygen sensor *in cellulo* (2). Moreover, PHD2 is the main enzyme required during embryonic development and PHD2 adult knockout mice suffer from severe polycythemia whereas PHD1 and PHD3 knockout mice were apparent healthy (3), (4). However, PHD1 knockout mice show changes in their cellular energy metabolism and PHD3 knockout mice have lower systemic blood pressure (4), (5). These observations may suggest that PHDs have redundant as well as non-redundant functions.

We wanted to explore the hypothesis if PHD2 has further hydroxylation substrates than HIF-α or if other regulatory mechanisms might be involved in the regulation of PHD2. We used yeast two-hybrid methology to identify novel PHD2 interacting proteins. We discovered that the FKBP38 protein binds specifically to PHD2 but not to PHD1 or PHD3. We performed a variety of interaction assays including yeast interaction analysis, GST pull-down and co-immunoprecipitation experiments, mammalian two-hybrid experiments and FRET analysis to confirm this interaction. The dissociation constant K_D determined by ITC measurements with about 0.9 μM suggests a relative strong interaction. Consistent with these data, calculations from FRET analysis with expressed fluorescent-tagged FKBP38 and PHD2 in HEK293 cells determined a distance between both proteins of about 5 nm which is even lower compared to the interaction of the hypoxically stabilized HIF-1α subunit and the constitutively expressed HIF-1β subunit with roughly 7 nm (6). Furthermore, we mapped the detailed interaction domain of FKBP38 with PHD2 and vice versa. FKBP38 binds with a linear binding motif from aa 37 to 56 to PHD2. The FKBP38 binding site is a so far unknown interaction motif containing a glutamate rich stretch. A shorter version of this motif can be found in mouse and rat.

PHD2 interacts with its N-terminal region from aa 1 to 114 with FKBP38. As a result of the peptide array and N-terminal deletion interaction analysis of PHD2 with FKBP38, we suggest that PHD2 binds with a non-linear binding region to FKBP38. The region from aa 1 to 114 contains the MYND-type Zn^{2+} finger domain named after its general structure in MTG8 and *drosophila* proteins *nervy* and DEAF-1 (7). It composes a cluster of cysteine and histidine residues arranged with an invariant spacing to form the potential zinc-binding motif. Currently it is believed that the MYND-type Zn^{2+} finger domain is a protein-protein interaction domain that provides a platform to recruit transcriptional repressors. Recent reports have suggested that PHD2 specifically represses HIF-1α transcriptional activity (8). Furthermore, the tumor suppressor protein ING4 interacts with PHD2 and recruits ING4 to HIF thereby limiting HIF transcriptional response by recruiting transcriptional repressors in hypoxia (9). More interestingly, PHD2 seems to shuttle between nucleus and cytosol (10). However, PHD2 localization has been mainly described to be in the cytosol but it does not exclude the possibility that a small portion of PHD2 might be also found in the nucleus. FKBP38 is localized at the mitochondria and ER and therefore interaction with PHD2 might be a way to keep PHD2 in the cytosol (11). Of note, a direct involvement of the MYND-type Zn^{2+} finger domain of PHD2 in FKBP38 binding has still to be determined by site-directed mutagenesis analysis.

FKBP38 is a member of the PPIase family. Even structurally unrelated, all members of this family have a common enzymatic activity that accelerates the *cis* to *trans* isomerization of the amino acid proline. It is thought that these enzymes play a role in protein folding and protein complex formation. We assumed that FKBP38 is a potential co-factor that plays a role in PHD2-mediated hydroxylation of HIF-α subunits. Though, stable RNA interference (RNAi)-mediated down-regulation of FKBP38 in HeLa cells increased PHD2 protein abundance and cellular hydroxylation activity. Genetic ablation of FKBP38 in MEFs slightly increased PHD2 protein as well. Conversely, elevated PHD2 protein levels delayed hypoxic HIF-1α protein accumulation and attenuated HIF-dependent transcriptional regulation. Reconstitution of FKBP38 normalized PHD2 protein levels and therefore cellular hydroxylation capacity and HIF-1 response. Strikingly, increased PHD2 protein abundance was due to prolonged PHD2 protein stability. These data suggest that FKBP38 determines PHD2 protein levels.

PHD2 transcript levels are induced in hypoxia by HIF, this induction might be regulated by TGFβ (12), (13). PHD2 activity is modulated by a wide range of components including oxygen, energy metabolism intermediates, iron and divalent ions, NO, ROS and antioxidants (14). However little is known about PHD2 protein regulation. PHD1 and PHD3 have been reported to be targeted by the E3 ubiquitin ligases Siah1/2 and subsequently degraded by the ubiquitin-proteasome pathway (15). PHD2 interacted with Siah1/2 but the functional consequence of this interaction remained elusive.

Protein synthesis and protein degradation are in a dynamic equilibrium that is important to control protein mass in a cell. The protein degradation pathways in the lysosomes and proteasomes account for 80% or more of the degradation of cellular proteins (16).

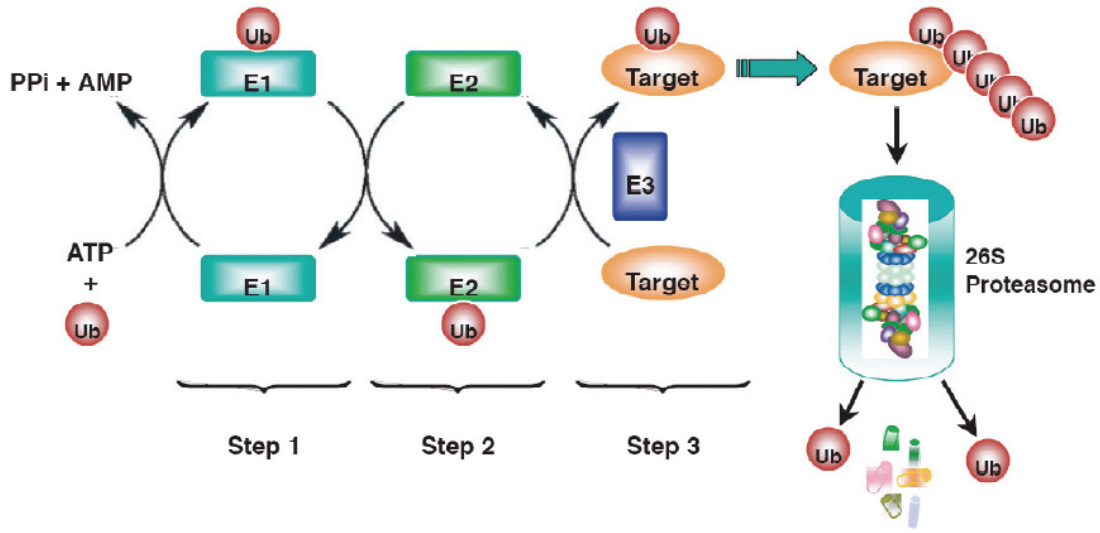

Figure 11. Scheme of the ubiquitin-proteasome degradation pathway (17)

Ubiquitin is first activated by the ubiquitin-activating enzyme E1 and then transferred to a member of the ubiquitin-carrier family of enzymes E2 (ubiquitin-conjugating enzyme). In the final reaction ubiquitin is added to the targeted protein with the help of an E3 ubiquitin ligase. Additionally, further ubiquitin moieties are added to generate a poly-ubiquitin chain and subsequently, the targeted protein is degraded in the 26S proteasome.

In the proteasome-ubiquitin system the protein substrate is tagged by covalent attachement of multiple ubiquitin molecules to generate a polyubiquitin chain (Fig. 11) (17). This polyubiquitin chain is the recognition marker for the degradation by the 26S proteasome complex and subsequently free and reusable ubiquitin and peptides are released from the 26S proteasome. The 26S proteasome consists of the 20S catalytic core protease particle and two regulatory 19S particles which cap the end of the 20S proteasome. Short-lived proteins such as p53, p73, ornithine decarboxylase that are inherently unstable are proposed to be degraded by default by the 20S

proteasome (18), (19). This degradation pathway does not require the ubiquitin-tag as recognition marker. Though these proteins can be stabilized by binding to the proliferating cell nuclear antigen (PCNA), antizyme or to the NAD(P)H quinone oxidoreductase 1 (NQO1) protein and prevent ubiquitin-independent proteasomal degradation (20).

We did not find an involvement of the ubiquitin-proteasome pathway in the degradation of PHD2 protein. Changes of PHD2 protein levels were not observed when MCF-7 cells were incubated with proteasomal inhibitors like MG132 or lactacystin. Lactacystin has been proposed to act as a pseudosubstrate that is covalently linked to the hydroxyl groups on the active site threonine in the β-subunit of the 20S proteasome and MG132 as a peptide aldehyde is a potent transition state inhibitor of the chemotrypsine-like activity of the 20S proteasome (21). To completely exclude the possibility of ubiquitin-dependent PHD2 proteasomal degradation we made use of a mouse ts20 cell line that contains a temperature sensitive E1 activating enzyme (22). Incubation of these cells under permissive (34ºC) or non-permissive temperatures (39ºC) allowed us to monitor protein accumulation. Whereas the known ubiquitin-dependently degraded p53 protein strongly accumulated, no changes in PHD2 protein levels were observed. Simultaneous incubation of a mouse ts20 cell line, corrected with a non-temperature sensitive E1 activating enzyme (H38-5), did not result either in accumulation of p53 or PHD2. Therefore, we have no evidence that PHD2 degradation occurs by the proteasome degradation pathway. Interestingly, PHD2 differs from the other PHDs by its protein structure with its N-terminal extension containing the MYND-type Zn^{2+} finger domain. N-terminal deletion of PHD2 rendered PHD2 susceptible to degradation by Siah1/2 similar to the other PHDs and afterwards proteasomal degradation whereas full length PHD2 was not prone to proteasomal degradation (23). These data suggest that PHD2 N-terminal region determines PHD2 protein degradation independent of the 26S proteasome.

Another well-established pathway of intracellular protein degradation involves the lysosomal pathway. Lysosomes contain a large variety of hydrolytic enzymes that degrade proteins or other substances taken into by endocytosis. Furthermore, whole organelles or large areas of cytoplasm can form so called autophagic vacuoles which fuse with lysosomes or late endosomes to degrade its content (24). Lysosomes have a low internal pH and proteins are degraded by the enzymatic activity of cathepsins

(cysteine proteases), aspartyl proteases and zinc proteases. Incubation of MCF-7 cells with a variety of common protease inhibitors like ABSF (serine protease inhibitor), E64 (cysteine protease inhibitor), ALLM (calpain inhibitor) and pepstatin A (aspartyl protease inhibitor) did not alter PHD2 protein levels. E64 an inhibitor of the lysosomal pathway did not affect PHD2 protein levels excluding the lysosomes as the organelle for PHD2 protein degradation. To entirely eliminate the possibility of the involvement of autophagy of organelles like ER and mitochondria in PHD2 protein degradation, cells should be incubated with the well-established macroautophagic inhibitor 3-MA (3-methyladenine) and PHD2 levels analyzed (25). Surprisingly, incubation of the cells with 2 mM EDTA, but not EGTA strongly reduced PHD2 protein levels though the physiological relevance is completely unclear (data not shown). These data suggest that PHD2 protein abundance is uniquely modulated.

The FKBP38 protein structure consists of a PPIase enzymatic activity domain, three TPRs, a CaM binding site and a transmembrane domain. FKBP38 has been demonstrated to be a Ca^{2+}-activated peptidyl prolyl *cis/trans* isomerase in *in vitro* protease-coupled *cis/trans* isomerase assays (26). Like full length FKBP38, deletion of the PPIase domain of FKBP38 enhanced the HIF-1α one-hybrid stability in MCF-7 cells and reconstituted it in the FKBP38-depleted cells 2G8 and 3D6. Furthermore, incubation of these cells with the FKBP38 specific inhibitor GP1046 and DM-CHX did not impair PHD2 protein levels. Hence, we concluded that FKBP38 modulates PHD2 protein abundance in a PPIase-independent manner.

FKBP38 possesses a transmembrane domain that anchored FKBP38 to mitochondria and ER. We questioned if the transmembrane domain of FKBP38 is required for PHD2 protein regulation. Surprisingly, loss of FKBP38 transmembrane did not reconstitute HIF-1α one-hybrid stability and PHD2 protein abundance in FKBP38-silenced cells. Strikingly, deletion of FKBP38 transmembrane domain abolished the binding to PHD2 though the PHD2:FKBP38 interaction is a prerequisite for PHD2 protein degradation. So far, fluorescent tagged PHD2 was localized in the cytosol but no further sub-cellular localization was described (27). We could detect PHD2 together with FKBP38 in the membrane fraction containing ER and mitochondrial proteins. Consistent with these data, in primary rat hepatocytes PHD2 mitochondrial staining was observed (28). Mitochondria are the cellular power plants because they supply the cells with ATP required for maintenance of the cellular metabolism. It is well established that mitochondria are also a major source for the

production of ROS. Inhibition of mitochondrial activity impaired HIF-1α accumulation whereas the molecular mechanism is controversially discussed (29), (30). Increased oxidative stress impaired PHD activity, however until now PHD2 protein stability was not investigated so far (31). Increased oxidative stress is associated with accumulation of oxidized proteins and with cellular aging. Mitochondria feature a ubiquitous enzymatic system that can reduce oxidized proteins or target oxidized proteins to mitochondrial matrix proteolytic enzymes for degradation (32). The ATP-stimulated mitochondrial matrix protease Lon degrades selectively the oxidized form of the mitochondrial aconitase enzyme (33). We speculate that the interface between FKBP38 and PHD2 at the mitochondria might make PHD2 protein susceptible to oxidation and degradation. Furthermore, during hydroxylation of HIF-α subunits PHD2 might be prone to oxidation. Though, PHD2 protein stability will be investigated in respiration-deficient cells (rho cells) or cellular respiration will be blocked with rotenone or myxothiazol or oxidative stress will be induced by hydrogen peroxide.

Additionally, FKBP38 has been described to have chaperone function in *in vitro* refolding assays (34). However, the region containing the FKBP38 chaperone activity has not been described so far. Chaperone proteins have a role in the refolding of misfolded proteins and not successful folding led to protein degradation. It would be of interest to investigate a possible role of FKBP38 chaperone activity in PHD2 protein regulation.

In summary, we discovered that FKBP38 specifically interacts with the oxygen-sensing prolyl-4-hydroxylase PHD2 thereby modulating the protein steady state levels in a PPIase-independent manner. The interaction of FKBP38:PHD2 requires the sub-cellular localization of FKBP38 at the ER and mitochondria and is the prerequisite for the regulation of the half-life of PHD2. So far, we were not able to identify the molecular mechanism of PHD2 protein degradation. We speculate that a unique protease or degradation pathway at the ER or mitochondria might be involved in the PHD2 breakdown. Disrupting the PHD2:FKBP38 interaction might be an attractive strategy to enhance PHD2 protein levels and to modulate the oxygen-sensing pathway.

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10 Curriculum vitae

PERSONAL INFORMATION

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EDUCATION

Since Febr. 2005	***PhD thesis*** Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich (Switzerland), Prof. R.H. Wenger and Dr. G. Camenisch "Functional characterization of the interaction of PHD2 with FKBP38"
Apr. 2004 - Dec. 2004	***Diploma thesis (Master thesis)*** Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich (Switzerland), Dr. G. Camenisch "Novel interaction of the oxygen-sensing prolyl-4-hydroxylase 2 (PHD2) with the prolyl *cis/trans* isomerase FKBP38"
Oct. 1999 - Dec. 2004	***Diploma studies in Human Biology (Bachelor and Master studies)*** Ernst-Moritz-Arndt-University of Greifswald (Germany) Major subject: Pharmacology; Minor subjects; Immunology, Molecular Biology
1992 - 1999	***Abitur (High school degree)*** Samuel-von-Pufendorf-Gymnasium Flöha (Germany)
1996 - 1997	***Participant of a High School Exchange Program*** Santa Fe Highschool Edmond / Southwest Covenant Highschool Oklahoma City (USA)
1986 - 1992	***Primary school*** Augustusburg/Germany

PROFESSIONAL EXPERIENCE AND TRAINING

Apr. 2003 - Aug. 2003	Scientific assistant in the group of Prof. S.B. Felix, Clinic for Internal Medicine, Ernst-Moritz-Arndt-University of Greifswald (Germany)
Oct. 2002 - Feb. 2003	Trainee in the Drug Discovery Department of Actelion Pharmaceuticals Ltd. Allschwil (Switzerland)
Aug. 2000	Internship in health care and nursing, Medical Center Zschopau (Germany)

SKILLS

Languages:	German (native), English (fluent), Russian, French (basic knowledge)
Software:	MS Office, GraphPadPrism4, CorelDRAW X3, Clone Manager
Teaching:	Assistance in practical courses in physiology for medical and human biology students (2005-2007)

COMMUNITY ACTIVITIES

2001 - 2003	Head and co-worker of international fellowship of evangelical student (IFES) group Greifswald, Germany
1998 - 1999	Assistant head of gymnastics group Augustusburg, Germany

CONGRESSES AND PRESENTATION

15.01. - 20.01.2008	Keystone Symposia Meeting " Molecular, Cellular, Physiological, and Pathogenic Responses to Hypoxia", Vancouver (Canada) - ***poster presentation***
29.05.2007	Seminar - Institute of Physiology, University of Zürich (Switzerland) - ***oral presentation***
25.03. - 28.03.2007	86th Annual Meeting of the German Physiological Society (DPG), Hannover (Germany) - ***oral presentation***
22.09.2006	2nd Zürich Center for Integrative Human Physiology (ZIHP) Symposium, Zürich (Switzerland) - ***poster presentation***
18.10.2005	Collaborative workshop with Bayer Healthcare AG, Wuppertal (Germany) - ***oral presentation***
06.10.2005	Annual Meeting of the Swiss Physiological Society (SwissPhysio), Bern (Switzerland) - ***oral presentation*** (Asher Hess Prize)
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GRANTS

04.02.2008	Travel Grant of the Swiss Physiological Society (CHF 1400.--)
27.05.2008	Swiss National Science Foundation postdoctoral fellowship for prospective researchers (1 year salary)

11 List of publications

Peer-reviewed original publications:

1. Jantzen F., Könemann S., Wolff B., ***Barth S.***, Staudt A., Kroemer H.K., Dahm J.B., Felix S.B., Landsberger M. (2007) Isoprenoid depletion by statins antagonizes cytokine-induced down-regulation of endothelial nitric oxide expression and increases NO synthase activity in human umbilical vein endothelial cells. **J Physiol Pharmacol**; 58(3):503-14
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3. ***Barth S.***, Nesper J., Hasgall P.A., Wirthner R., Nytko K.J., Edlich F., Katschinski D.M., Stiehl D.P., Wenger R.H. and Camenisch G. (2007) The peptidyl prolyl cis/trans isomerase determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. **Mol Cell Biol**; 27(10):3758-68
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Publication with limited peer-review:

1. Wirthner R., Kuppusamy B., Stiehl D.P., ***Barth S.***, Spielmann P., Oehme F., Flamme I., Katschinski D.M., Wenger R.H., Camenisch G. (2007) Determination and modulation of prolyl-4-hydroxylase domain (PHD) oxygen sensor activity. **Methods Enzymol**; 435:43-60

Manuscripts in preparation:

1. ***Barth S.***, Edlich F., Berchner-Pfannschmidt U., Gneuss S., Shirane M., Wenger R.H. and Camenisch G. (2008) HIF prolyl-4-hydroxylase 2 (PHD2) is modulated by its FKBP38-dependent subcellular localization.
2. Landsberger M., Jantzen F., Könemann S., Wolff B., ***Barth S.***, Staudt A., Kroemer H.K., Felix S.B. (2008) Statins up-regulate LOX-1 expression but reduce uptake of oxidized LDL in cytokine-treated human endothelial cells.
3. Balamurugan K., Hofmann V.S., Stiehl D.P., ***Barth S.***, Wenger R.H., Camenisch G. (2008) Onconeuronal cerebellar degeneration-related antigen Cdr2 interacts with prolyl hydroxylase 1 and suppresses the function of hypoxia-inducible factor.

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